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COMPARISON OF THE TUMOURS PRODUCED BY 2-ACETYL-AMINO-FLUORENE IN PIEBALD AND WISTAR RATS.

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DIFFERENT strains of a species exposed to the action of the same carcinogen can show considerable variations in susceptibility and in tissue responses. Andervont and his collaborators (1942, 1943) by using pure strains of mice demonstrated the importance of genetic factors in the pathogenesis of tumours induced with ortho-amino-azotoluene and 3 : 4 : 5 : 6-dibenzcarbazole (1941). Strong (1945, 1945*a*) has developed strains of mice which react to injected methylcholanthrene with the development of distant tumours. It was therefore not surprising that variations in tissue responses were found in two strains of rats which received 2-acetyl-amino-fluorene *per os*.

In a previous paper (Bielschowsky, 1944) the localization of distant tumours induced by 2-acetyl-amino-fluorene in a sub-strain of Wistar rats has been recorded. Here the results obtained by the same method in a strain of piebald rats will be given.

METHODS.

Piebald rats, 6–7 weeks old, were given 4 mg. of 2-acetyl-amino-fluorene daily for 25 weeks *per os*. Animals which died during the first weeks of the experiment and some which were lost because their cadavers were eaten by their mates are omitted from the report. Only rats which received the full dose of the carcinogen or developed tumours in less than 175 days are included. The animals were killed as soon as the presence of a tumour could be established.

RESULTS.

Tables I and II give in chronological order the post-mortem findings in male and female piebald rats. Three types of neoplasms occur with great frequency in this strain: (1) Tumours of the ductus acousticus externus. (2) Hepatomas and benign cystic cholangiomas. (3) Adenocarcinomata of the small intestine. There are no striking differences in the frequency of these neoplasms in the two sexes. Comparison of these results with those obtained in our inbred strain of Wistar rats revealed three main dissimilarities. (1) Only one of the 25 females

of the piebald strain developed a mammary cancer as compared with the incidence of more than 60 per cent in the female Wistar rats. (2) More than 50 per cent of the piebald rats had tumours of the small intestine, whereas the frequency of such neoplasms in our Wistar rats, receiving the same acetyl-amino-fluorene-containing diet, is low. Tumours of the jejunum or ileum have so far been found in 7 of 133 males and 11 of 166 females of this strain. This difference applies only to tumours of the small intestine, but not to those of the colon. Two carcinomata of the colon have been found in the 38 piebald rats and 14 in 300 Wistar rats. (3) The tumours of the ductus acousticus externus are much more frequent in the piebald than in the Wistar rats.

TABLE I.—*Male Piebald Rats.*

	Duration of experiment.	Hepatomas.*	Tumours of ductus acousticus externus.	Tumours of small intestine.	Tumours in other organs.
1	141 days	+	+	0	..
2	169 ,,	+	+	0	Carcinoma of lung.
3	173 ,,	+	+	0	..
4	175 ,,	0	+	0	..
5	181 ,,	+	++	+	..
6	188 ,,	+	0	0	..
7	195 ,,	0	++	+	..
8	197 ,,	+	0	0	Leukaemia.
9	204 ,,	+	0	+	..
10	208 ,,	+	+	+	..
11	208 ,,	+	++	+	..
12	211 ,,	0	+	+	..
13	230 ,,	+	+	0	..

0 = No tumour. + = One tumour. ++ = Two tumours.

* Benign cystic cholangiomata, present in nearly all animals, are not listed.

The liver tumours induced by acetyl-amino-fluorene are of similar histological structure to the liver tumours obtained in rats with dimethyl-amino-azobenzene, and which have been admirably described by Opie (1944).

Fig. 1–4 show the development of the tumours of the ductus acousticus externus. The photomicrographs illustrate the changes from adenoma to squamous keratinizing carcinoma, which in its later stages frequently invades the muscle and sometimes metastasizes.

The Tumours of the Small Intestine.

The great majority of these neoplasms are situated ten to eighteen inches below the pylorus. They originate more often on the side of the intestine opposite to the mesentery, and are found sometimes in close vicinity to Peyer's patches. Two tumours of the small intestine were found in each of six piebald rats, one situated in the jejunum, the other in the ileum with an interval of several inches. Multiple intestinal tumours have been seen only once in the albino

rats. Complete ileus or formation of a faecal fistula was never observed. Twice, however, a tumour was the cause of an intussusception which did not produce symptoms of acute illness. Signs of incomplete ileus, dilatation of the loop proximal to the tumour and stasis of its faecal content were seen when the tumours had reached considerable size. Such animals frequently suffered from diarrhoea.

TABLE II.—*Female Piebald Rats.*

	Duration of experiment.	Hepatomas.*	Tumours of ductus acousticus externus.	Tumours of small intestine.	Tumours in other organs.
1	174 days	0	+	++	..
2	174 „	0	+	+	..
3	176 „	0	++	0	..
4	188 „	0	++	0	..
5	195 „	+	+	0	..
6	202 „	0	+	+	Caecum.
7	202 „	0	+	+	..
8	209 „	+	++	0	Caecum.
9	209 „	+	+	+	..
10	215 „	0	+	++	..
11	218 „	0	++	+	..
12	218 „	0	+	0	Adenoma of lung.
13	220 „	0	+	0	..
14	230 „	0	+	+	..
15	237 „	0	+	0	..
16	237 „	+	+	0	Adenoma of lung.
17	237 „	0	+	+	..
18	238 „	+	+	+	..
19	246 „	+	0	+	..
20	251 „	+	+	+	..
21	281 „	0	0	0	..
22	293 „	+	+	++*b*	Mammary cancer.
23	293 „	0	0	0	Cancer of eyelid.
24	293 „	+	0	0	..
25	293 „	+	0	+*b*	..

b = Benign neoplasma.

* Benign cystic cholangiomata, present in nearly all animals, are not listed.

The histological study of these tumours revealed that in all but two instances the neoplastic process had involved all the layers of the gut. Only one of the intestinal tumours found in rat 22 and the one in rat 25 (Table II) were limited to the mucosa (Fig. 5.) The photomicrograph shows a sessile papilloma, the epithelium of which is clearly distinct from the normal epithelium of the mucosa. Invasion of the submucosa and muscularis occurred at an early stage; it was

already present in small tumours having a diameter of 3–5 mm. The glandular structure remained generally well preserved, but became more and more distorted the deeper the adenocarcinomata invaded the outer layers of the gut. Cystic dilatation of such neoplastic glands was common, and could reach dimensions surpassing the diameter of the lumen of the intestine. In the small intestine the connective tissue frequently reacted against the invading tumour with the formation of a new wall so that macroscopically the impression of a knoblike protuberance was produced. Fig. 8 shows a typical example of such structure. Here the greater part of the tumour consists of fibroblasts surrounding the cystic adenocarcinoma. In one of the larger adenocarcinomata of the jejunum, osteoid was found in such newly formed connective tissue (Fig. 10). The transition from normal to neoplastic epithelium was generally abrupt, and the more basophilic tumour cells were easily distinguishable. Although in many instances the two main types of cell lining the intestinal canal, the mucus-forming and the columnar epithelium, were still found together in the adenomatous tumour, there were instances where one type predominated over the other (in Fig. 6 the clear mucus-forming cells and in Fig. 10 the columnar cells). Paneth cells were not found to be a conspicuous cell element in these neoplasms, but no special stainings were used to trace these cells.

As mentioned above, 16 neoplasms of the colon have been found in rats treated with 2-acetyl-amino-fluorene. They resembled in many aspects the neoplasms of the small intestine and seemed to begin as papillomata (Fig. 11 and 13). The lateral wall of the caecum was the site of predilection. One tumour of the rectum prolapsed through the anus, and provided the only instance where the diagnosis of an intestinal tumour was made *in vivo*. There was one distinguishing feature: the invasion of the submucosa and the muscularis occurred only when the tumours had reached a considerable size and the reaction of the connective tissue to the invading cancer cells was less conspicuous.

Experience with a great number of rats treated with 2-acetyl-amino-fluorene has shown that the longer a rat lives, the greater is the chance for the development of the less frequent types of tumours. It was thought to be of greater importance to establish when and in which order acetyl-amino-fluorene induces malignant lesions in various organs, than to collect the greatest possible number of neoplasms. By killing the animals as soon as the presence of a tumour could be established, it could be shown that in the male piebald rats hepatomas develop at the same rate as the tumours of the ductus acousticus externus, whereas in

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DESCRIPTION OF PLATES.

FIG. 1.— × 22. Early tumour of ductus acousticus externus.
FIG. 2.— × 90. Detail from Fig. 1 = A.
FIG. 3.— × 90. Detail from Fig. 1 = B.
FIG. 4.— × 90. Detail from Fig. 1 = C.
FIG. 5.— × 7. Papilloma of the small intestine.
FIG. 6.— × 90. Detail from Fig. 5.
FIG. 7.— × 7. Early adenocarcinoma of the small intestine.
FIG. 8.— × 16. Adenocarcinoma of the small intestine.
FIG. 9.— × 7. Advanced adenocarcinoma of the small intestine.
FIG. 10.— × 90. Detail from Fig. 9.
FIG. 11.— × 7. Papilloma of caecum.
FIG. 12.— × 90. Detail from Fig. 11.
FIG. 13.— × 7. Papilloma of colon.
FIG. 14.— × 7. Adenocarcinoma of colon.

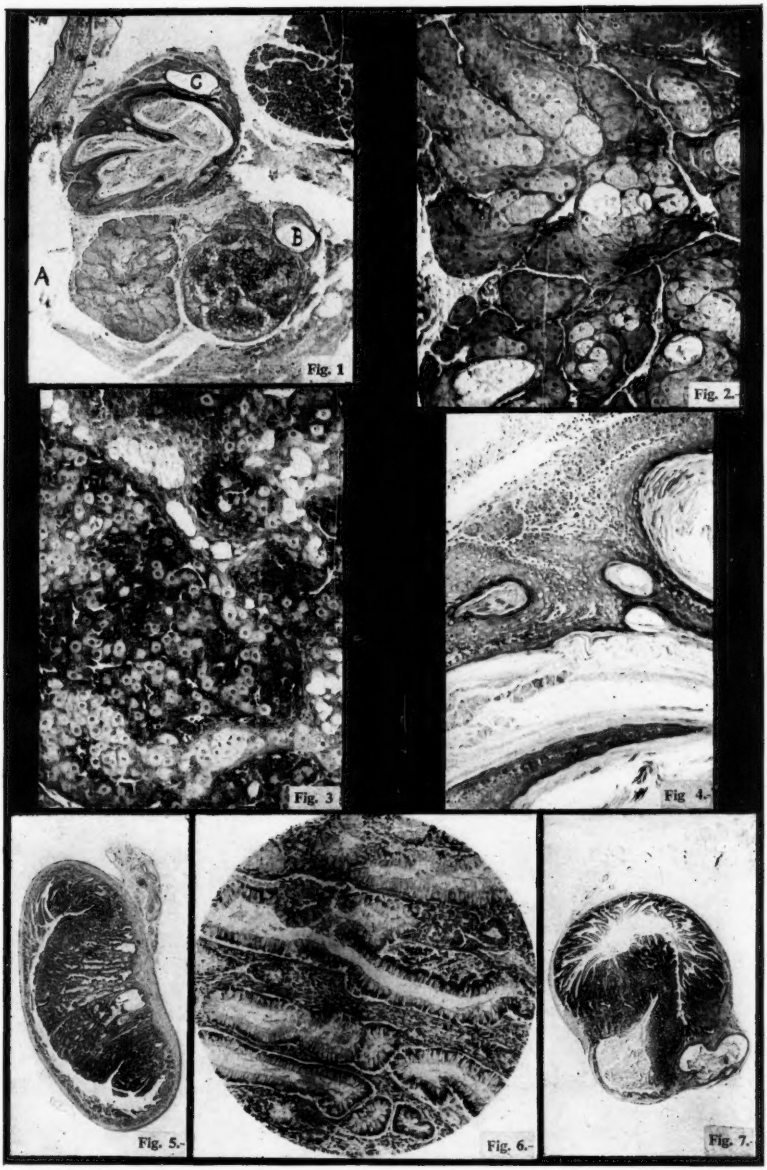

Fig. 1

Fig. 2.

Fig. 3

Fig 4.

Fig. 5.

Fig. 6.

Fig. 7.

Bielschowsky.

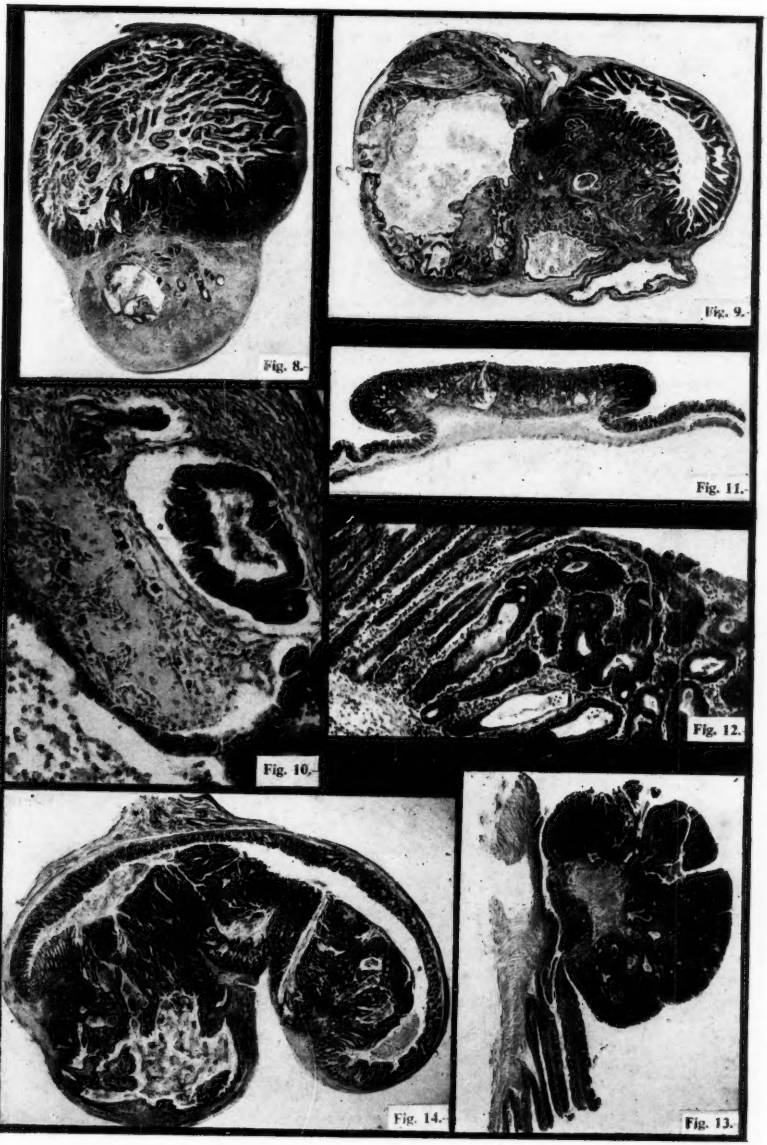

Fig. 8.

Fig. 9.

Fig. 11.

Fig. 12.

Fig. 10.

Fig. 14.

Fig. 13.

the females intestinal tumours and cancers of the ductus acousticus externus tend to appear first. The frequency of the liver tumours in females would probably have risen considerably if these animals had been allowed to live for several more weeks.

DISCUSSION.

Tumours of the small intestine have been produced in mice by Lorenz and Stewart (1940) following oral administration of 1 : 2 : 5 : 6-dibenz-anthracene and 20-methyl-cholanthrene. Carcinogenic hydrocarbons usually act at the site of application, and induce neoplastic changes in many tissues of mice, when the experimental conditions are such that susceptible cells are exposed to their action long enough. It has not been decided whether 2-acetyl-amino-fluorene acts by a similar mechanism on the mucosa of the intestine, or if the cancers of the gut are to be counted among the distant tumours induced by this compound in many organs of rats and mice. Such a conception would suggest the existence of an " active " metabolite, formed either in the liver and then excreted with the bile or by enzymatic processes within the lumen of the gut, or by the action of the intestinal flora. Experiments are in progress designed to elucidate this question.

The literature of distant cancers of the breast of the rat induced by compounds of known chemical structure, and having no oestrogenic action, is scanty. But there exist several strains of mice in which such neoplasms can be obtained with methyl-cholanthrene. Strong (1945) has recently reviewed the literature on this subject. It seems certain that factors other than Bittner's milk factor are determinant for the susceptibility of this organ to non-oestrogenic carcinogens. How far such differences are due to endocrine factors is unknown.

SUMMARY.

The distribution of neoplasms induced by 2-acetyl-amino-fluorene in a strain of piebald rats is recorded, and a description is given of the intestinal tumours which are found frequently in this strain but only rarely in our strain of Wistar rats. Cancer of the breast was found only once in a piebald rat.

My thanks are due to Prof. H. N. Green for his interest and encouragement, to Mr. O. Thornburn for his competent technical assistance, and to Mr. A. W. Collins for the preparation of the photomicrographs.

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INVESTIGATION INTO THE PRODUCTION OF BACTERIOSTATIC SUBSTANCES BY FUNGI. PRELIMINARY EXAMINATION OF THE FIFTH 100 SPECIES, ALL BASIDIOMYCETES, MOSTLY OF THE WOOD-DESTROYING TYPE.

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THIS work is a continuation of that already published in the series (Wilkins and Harris, 1942, 1944; Wilkins, 1945), which has dealt with an examination of some 1100 fungal species for the production of antibacterial substances.

The present paper deals with the examination of 100 wood-destroying Basidiomycetes isolated in culture, grown on solid and liquid media and tested comparatively by the " strip " method (Wilkins and Harris, 1944*b*) and the " hole " method (Wilkins and Harris, 1943*a*) against *Bacterium coli* and *Staphylococcus aureus*.

The cultures used in this work were obtained from the Forest Products Research Laboratory of the Department of Scientific and Industrial Research by kind permission of the Director, Dr. F. Y. Henderson, and with the co-operation of the Mycologists, Mr. K. StG. Cartwright and Dr. W. P. K. Findlay. For purposes of identification of the actual strain used, the Research Laboratory number is given after the name of each species.

The fungi were first grown in petri dishes in three agar media, viz. malt, potato dextrose and modified Czapek-Dox. When the colony had reached a suitable diameter the fungus was tested by the " strip " method. In the case of those giving a positive result, the method indicated which part of the colony was most active in the production of antibacterial substances, and from this region on the most favourable medium, discs 11 mm. in diameter were cut and floated on the surface of 10 ml. of the same three media, this time liquid, in flasks. The metabolism solution was tested by the " hole " method after five days and at subsequent intervals of 5 days up to 30 days. In the case of fungi giving a negative result by the " strip " method discs were cut near the growing margin of the colony, and these were also grown on the three liquid media and similarly tested. In all cases incubation was at 25° C. Unexpected positive results against *Bact. coli* with the Czapek-Dox medium caused this medium to be regarded with suspicion, and tests showed that the sodium ion of the sodium nitrate was itself slightly bacteriostatic. The Czapek-Dox was therefore discontinued.

The results listed below, which were obtained by both the " strip " method

and the liquid method, are divided into three sets : (1) Those fungi which by the liquid method gave an inhibition area of more than 20 mm. against at least one of the bacteria ; (2) those which by the liquid method gave an area of more than 15 mm. ; and (3) those which gave an inhibition area of less than 15 mm. or none at all. The first two lists are described respectively as " strongly positive " and " weakly positive," the last as " negative." Only strongly positive have been written out in detail for discussion purposes, and the figures represent the diameter of the area of inhibition in mm. When two figures appear in the time column, e.g. 15/23, it means that the maximum inhibition was obtained in 15 days on the first medium (malt) and in 23 days on the second medium (P. dextrose).

LIST OF FUNGI EXAMINED.

Strongly Positive.

	Strip.			Liquid.		
	Time (days).	Malt.	P.D.	Time (days).	Malt.	P.D.
Coniophora cerebella Pers. 11A	6	7.9	.14	30/10	25.26	15.15
Cyathus striatus (Huds.) Pers. 22	11	.8	.10	25/30	.24	.16
Daedalea quercina (Linn.) Fr. 38	16	—	8.8	10/30	.16	21.21
Fomes annosus Fr. 41B	10	14.20	11.21	10	20.25	15.18
Lenzites saepiaria (Wulf.) Fr. 10	12	15.19	.10	20/15	16.23	16.19
Merulius lachrymans (Wulf.) Fr. 12	12	9.10	12.15	20	.20	16.23
M. tremellosus (Schrad.) Fr. 13	9	.9	.8	10	.23	.20
Polyporus betulinus (Bull.) Fr. 4	16/20	20.18	12.14	10/15	31.29	21.20
P. eucalyptorum Fr. 257	20	8.16	8.16	25	16.22	17.23
Poria ferruginea-fusca Karst. 193	14	.12	.8	15/20	.22	.23
P. vaillantii (D.C.) Fr. 14	9/14	..	.6	20	.22	17.21
P. xanthia Lind. 62	10	8.8	14.16	15	21.19	20.21

Weakly Positive.

Clitocybe aurantiaca (Wulf.) Studer. 207 ; *Collybia velutipes* (Curt.) Fr. 54 ; *Fomes annosus* Fr. 41 ; *F. lignosus* Klotzsch. 202 ; *Hypholoma fasciculare* Huds. 32 ; *H. sublateritium* Schaeff. 58 and 58A ; *Lentinus lepideus* Fr. 7D ; *Lenzites striata* Swartz. 204 ; *Panus stipticus* (Bull.) Fr. 39 ; *P. torulosus* (Pers.) Fr. 64 ; *Polyporus Mikadoi* Lloyd. 226 ; *P. ochroleucus* Berk. 261 ; *P. radiatus* (Sow.) Fr. 37 ; *P. rugulosus* Lév. 214 ; *P. stipticus* (Pers.) Fr. 208 ; *P. zonalis* Berk. 216 ; *Poria medulla-panis* Pers. 258 ; *P. vaillantii* (D.C.) Fr. 57 ; *Stereum hirsutum* Fr. 3 ; *S. spadiceum* Fr. 69A ; *Trametes Feei* Fr. 201.

Negative.

Armillaria mellea (Vahl.) Fr. 6 ; *A. mucida* (Schrad.) Fr. 5 ; *Collybia fusipes* (Bull.) Berk. 55 ; *C. radicata* (Relh.) Berk. 244 ; *Daedalea flavida* Lév. 252A ; *Daldinia concentrica* (Bolt.) Ces. et de Not. 26A ; *Fistulina hepatica* (Huds.) Fr. 67B and 67C ; *F. ferruginosus* (Schrad.) Massee. 47A ; *F. fraxineus* (Bull.) Fr. 17 and 17A ; *F. geotropus* Cke. 221 ; *F. marmoratus* (B. et C.) Cke. 223 ; *F. noxius* Corner. 224A ; *F. pomaceus* (Pers.) Big. et Guill. 33A ; *F. ribis* (Schum.) Fr. 42 ; *F. ulmarius* (Sow.) Fr. 241 ; *Ganoderma applanatum* (Pers.) Pat. 20 ; *Hydnum coralloides* (Scop.) Fr. 60 ; *Hymenochaete rubiginosa* (Dicks.) Lév. 243 ; *Hypoxylon coccineum* Bull. 209 ; *Lentinus tigrinus* (Bull.) Fr. 68 ; *Lenzites betulina* (Linn.) Fr. 34 and 34A ; *L. tricolor* (Bull.) Fr. 235 ; *Merulius confluens* Schw. 220 ;

M. serpens (Tode) Fr. 15A ; *Phellinus cryptarum* Karst. 178B ; *Phlebia merismoides* Fr. 19 ; *Pholiota adiposa* Fr. 52A ; *P. mutabilis* (Schaeff.) Quél. 49 ; *Pleurotus euosmus* (Berk.) Cke. 66 ; *P. ostreatus* (Jacq.) Fr. 40 ; *Polyporus adustus* Fr. 35 ; *P. dryophilus* var. *vulpinus* (Berk.) Fr. 183 ; *P. fumosus* (Pers.) Fr. 24 ; *P. hispidus* (Bull.) Fr. 43 ; *P. intybaceus* Fr. 36 ; *P. picipes* Fr. 186 ; *P. pubescens* (Schum.) Fr. 187 ; *P. radiatus* (Sow.) Fr. 37B ; *P. Spraguei* B. et C. 227 ; *P. sulphureus* (Bull.) Fr. 29 ; *P. tenuis* Sacc. 228 ; *P. tsugae* (Murr.) Overholt. 189 ; *P. tulipiferus* (Schwein.) Overholt. 191 ; *P. velutinus* Fr. 229 ; *P. zonatus* (Nees) Fr. 199 ; *Polystictus abietinus* (Dicks.) Fr. 2 ; *P. cinnabarinus* (Jacq.) Cke. 176 ; *P. hirsutus* (Wulf.) Fr. 1 ; *P. occidentalis* (Klotzsch.) Fr. 230 ; *P. pergamenus* Fr. 254 ; *P. versicolor* (Linn.) Fr. 28 ; *Poria obducens* (Pers.) Fr. 51A ; *P. punctata* (Fr.) Cke. 195 ; *Schizophyllum commune* Fr. 9 ; *Stereum gausapatum* Fr. 226 ; *S. pini* Fr. 267 ; *S. rugosum* (Pers.) Fr. 61 ; *S. sanguinolentum* (Alb. et Schw.) Fr. 27 ; *Trametes gibbosa* (Pers.) Fr. 25 ; *T. isabellina* Fr. 255 ; *T. pini* (Brot.) Fr. 45A ; *T. rubescens* (A. et S.) Fr. 59 ; *Xylaria polymorpha* Grev. 18.

The results show that about 10 per cent are strongly and 20 per cent weakly positive. This is in accordance with expectation. The specific influence of the different media and the relative degree of bacteriostatic action against the two types of bacteria are sufficiently indicated to obviate the necessity for comment. It has been established that, as an initial positive/negative test, the strip method is reasonably determinative.

During the progress of this work an interesting paper by Robbins, Hervey, Ross, Ma and Robbins (1945) has appeared. This paper deals with fungi similar to those discussed here and results are similar, but detailed comparison will be postponed till later.

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SHOPE PAPILLOMA AND SHEEP DERMATITIS IN THE RABBIT: MUTUAL INTERFERENCE, SUPERINFECTION, AND EFFECTS OF CHEMICAL PRE-TREATMENT OF THE SKIN.

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It has been previously shown that the rabbit and guinea-pig are susceptible to infection by a virus which was originally derived from an outbreak of sheep dermatitis (Selbie, 1944, 1945). In the rabbit the infection produces a proliferative type of lesion involving the hair sheaths, and giving rise to growths that resemble papillomas. In the guinea-pig, on the other hand, the infection is more rapid in its evolution, and the lesion is of a degenerative type with the formation of vesicles and scabs (Selbie, 1946). The diversity of the manifestations of this virus infection in these two species of animals suggests the possibility that the type of lesion in a single species might be altered by modifying the receptivity of the infected cells. In the experiments to be described here two methods have been used with a view to altering the receptivity of the rabbit skin to this strain of sheep dermatitis virus. In the first method the skin is infected with Shope papilloma virus either at the same time or at an interval before or after inoculation with sheep dermatitis virus. In the second method the skin receives a preliminary treatment with a carcinogenic hydrocarbon or with croton oil, the latter of which is known to produce only a temporary hyperplasia of the epidermis. At the same time, parallel experiments have been carried out with the Shope papilloma, an infection which is known to respond to modification of the epidermal cells by pre-treatment with carcinogenic agents (Rous and Kidd, 1938; McIntosh and Selbie, 1940). Although these methods have produced no radical changes in the lesions of Shope papilloma and sheep dermatitis, the results have proved of considerable interest with regard to the phenomena of virus interference and superinfection.

MATERIAL AND METHODS.

Preparation of rabbits.

Twelve rabbits were used in these experiments, and they were prepared for chemical treatment or virus inoculation by plucking the fur from an anterior and posterior area of the skin of the right flank, designated Areas 1 and 2, and from corresponding areas of the left flank, designated Areas 3 and 4. Each area of epilated skin measured about 6 by 8 cm. and was prepared on the day preceding the commencement of chemical treatment or virus inoculation. The areas that

were treated with croton oil did not require further epilation before the inoculation of virus, but the areas receiving treatment with the carcinogenic agent, which lasted for 5 weeks, had to be clipped close on several occasions. The rabbits were allotted in groups of 4 to Expts. 1, 2 and 3, of which the details are shown in Tables I, II and III.

Chemical pre-treatment.

Carcinogenic hydrocarbon.—About 0·1 c.c. of a 0·5 per cent solution of 9 : 10-dimethyl-1 : 2-benzanthracene in benzene was spread over the area of skin by means of a fine bore pipette at each application. Six applications were made at intervals of one week, the last application being made 3 days before the date fixed for the inoculation of virus.

Croton oil.—A 0·3 per cent solution of croton oil in acetone was applied to the skin by the same method as that used for the carcinogenic agent. Six applications were made on alternate days, the last application being made on the day preceding the inoculation of virus.

Virus inoculations.

Infective suspensions of the viruses were prepared by grinding fresh or glycerolated lesions with sand and suspending in saline. The area of skin to be inoculated was scarified with a sharp needle to give a criss-cross pattern, and then four drops of the infective suspension was rubbed in with a match-stick. The Shope papilloma material was derived from a cottontail, and had been kept in 50 per cent glycerol for 7 years. In preparing the papilloma virus suspensions for the three experiments, weighed quantities of the glycerolated material were used so as to make the suspensions as nearly comparable as possible. The sheep dermatitis suspension was prepared from scrapings of fresh 18-day lesions from the 25th rabbit passage, and all the dermatitis virus inoculations in the three experiments were done with this supension on the same day in order to obviate uncertainties that might have arisen from inequalities in the infectivity of different dermatitis virus suspensions. In Expt. 3, where the two viruses were inoculated simultaneously, the two virus suspensions were mixed before inoculation and in such a manner that the final dilution of each virus was equal to that used in the single virus inoculations. When the two viruses were inoculated successively, the papilloma virus was inoculated 6 days after the dermatitis virus (Expt. 2) and the dermatitis virus 14 days after the papilloma virus (Expt. 1).

RESULTS.

Protocols of Experiments 1, 2 *and* 3.

Although there were some differences in the susceptibility of the rabbits to the viruses of Shope papilloma and sheep dermatitis infections, they were not sufficient to interfere with the interpretation of the results. Only one rabbit (Expt. 2, No. 8) showed a high degree of resistance, but this did not interfere with the experiment, because the rabbit was apparently equally resistant to both viruses, There was also a certain amount of variation in the growth of the Shope papilloma. as is usual with this infection, but the differences to be described here between the growth in different areas of skin of the same rabbit were greater than could be ascribed to normal variation.

Experiment 1: *Shope papilloma.*

In Expt. 1 all four areas of the skin of Rabbits 1 to 4 were inoculated with Shope papilloma virus on December 1, 1944. The further details of the treatment of Areas 2, 3 and 4 are shown in Table I. Rabbit 4 was killed accidentally

TABLE I.—*Expt.* 1 (*Rabbits* 1 *to* 4): *Virus Inoculations and Chemical Pre-treatment.*

Skin area.	Pre-treatment.	Virus inoculations.
1	None	Shope papilloma.
2	,,	Shope papilloma, followed at 14 days by sheep dermatitis.
3	9 : 10-Dimethyl-1 : 2-benzanthracene	Shope papilloma.
4	Croton oil	,, ,,

12 days after inoculation. In the remaining 3 rabbits papillomas were first observed in all 4 areas between the 18th and 22nd day, and in Area 2 dermatitis infection appeared 8 days after the inoculation of dermatitis virus. The various treatments of the skin had therefore no apparent effect on the length of the incubation period of either virus infection.

In Area 2 papillomas and dermatitis appeared at about the same time, but within a week the papillomas were difficult to distinguish, as they became merged with the diffuse thickening and irregular surface of the dermatitis lesion. After a further 7 to 10 days the papillomatous lesion became more distinct, but differed from the lesion in Area 1 in that it was divided into more numerous elements, while the individual papillomas were thinner and had a scurfy surface characteristic of dermatitis infection. In their later development the papillomas in this area remained thinner and had a more keratinized surface than in the untreated area for at least 17 weeks in Rabbits 1 and 3, and up to the death of Rabbit 2 at 74 days.

The dermatitis lesion in Area 2 was less severe and more rapid in its evolution than in normal rabbit skin; at no stage did it form the characteristic outgrowths of this infection, and all evidence of its presence had disappeared by the 35th day after the inoculation of dermatitis virus except for the modification that it had brought about in the appearance of the papillomas.

In Area 3, where the skin had been treated with the carcinogenic agent, the number of papillomas was only about half that of Area 1 and they were much slower in their growth. The smaller size and less fleshy appearance of the papillomas in this area was noticeable up to 7 to 10 weeks in Rabbits 1 and 2 and up to 10 months in Rabbit 3.

In Area 4, where the skin had been treated with croton oil, the crop of papillomas was about the same as in Area 1, but the papillomas were larger and had a fleshier appearance. This difference between the papillomas in Areas 1 and 4 remained distinctive up to about 9 to 12 weeks in Rabbits 1 and 2 and up to 10 months in Rabbit 3.

Histology.—The histological appearances of biopsies from the four areas of skin in Rabbit 2, 34 days after inoculation of papilloma virus, are shown in Fig. 1 to 4. Fig. 1, from Area 1, shows a typical Shope papilloma which, at this stage, is built up entirely above the epidermis and shows little tendency to extend

into the dermis. It is thus readily distinguished from the lesion of dermatitis which involves the hair sheaths within the dermis as shown in Fig. 5. In Fig. 2, from Area 2, it will be seen that there has been a slight suppression of the papillomatous lesion by the subsequent inoculation of dermatitis virus. Here the papillomas are more superficial and more keratinized, while the epithelial papillae are more elongated and less fleshy than in Fig. 1. It will also be noted that in this area, except for some thickening of the dermis, there is little evidence of dermatitis infection which, at this stage, would be fully developed on a normal area of skin, as is shown in Fig. 5. The effect on the papilloma virus infection of pre-treatment with the carcinogenic agent in Area 3 (Fig. 3) has been a reduction in the size and number of the papillomas, whereas croton oil pre-treatment in Area 4 (Fig. 4) has caused a denser and fleshier growth than in the untreated area of skin.

A section of Area 2, Rabbit 1, taken 49 days after the inoculation of papilloma virus shows that the dermatitis lesion has now regressed completely. The papillomas in this area still have a more elongated appearance than in Area 1 in sections from Rabbit 1 up to 101 days, in Rabbit 2 up to 74 days and in Rabbit 3 up to 306 days. The smaller size of the papillomas in Area 3, as compared with Area 1, can still be seen in sections from Rabbit 1 up to 101 days and in Rabbit 3 at 63 days, but not in Rabbit 2 at 74 days or in Rabbit 3 at 306 days. In Area 4 the larger size and more fleshy appearance of the papillomas, as compared with Area 1, is shown in sections from Rabbit 2 at 74 days and Rabbit 3 at 63 days, but not in Rabbit 1 at 49 days or Rabbit 3 at 101 days.

Experiment 2: sheep dermatitis.

In Expt. 2 all four areas of the skin of Rabbits 5 to 8 were inoculated with sheep dermatitis virus on December 15, 1944. The further details of the treatment of Areas 2, 3 and 4 are shown in Table II. The characteristic erythema

TABLE II.—*Expt. 2 (Rabbits 5 to 8), Virus Inoculations and Chemical Pre-treatment.*

Skin area.	Pre-treatment.	Virus inoculations.
1	None	Sheep dermatitis.
2	,,	Sheep dermatitis, followed at 6 days by Shope papilloma.
3	9 : 10-Dimethyl-1 : 2-benzanthracene	Sheep dermatitis.
4	Croton oil	,, ,,

and scaliness of the first stage of dermatitis infection could be seen in all four areas 8 days after the inoculation of dermatitis virus, but in Area 4, where the skin had been treated with croton oil, the lesion was more advanced and already showed some thickening of the skin. The further evolution of the lesions and the variations which occurred from area to area were similar in Rabbits 5, 6 and 7, but Rabbit 8 was relatively resistant to both virus infections, so that there was only a transient dermatitis infection and only a scanty crop of small papillomas appeared in Area 2. The following description will therefore be confined

to Rabbits 5, 6 and 7, although similar results were obtained in Rabbit 8, but in a much modified form.

The development of the dermatitis lesions in Areas 1 and 2 was apparently about equal until the nodular stage was reached about the 25th day. After this stage the effect of the papilloma infection on the dermatitis lesion in Area 2 was shown in the more rapid onset of regression which was completed by the 6th week, fully 2 weeks earlier than in Area 1.

The papillomas in Area 2 were at first obscured in the depths of the nodular dermatitis lesion, and did not become visible until at least four weeks after the inoculation of papilloma virus, when the dermatitis nodules began to separate and fall off. For a further 6 to 10 weeks the papillomas were smaller and more keratinized than in the untreated skin of Area 1 in the rabbits of Expt. 1, but in their later development this difference was no longer noticeable. It was also noted that the papillomas in this area were smaller than those in Area 2 of Expt. 1, so that it was apparent that the papillomatous infection was more reduced by a preceding dermatitis virus infection than by the subsequent inoculation of dermatitis virus.

In Area 3, which had been treated with the carcinogenic agent, the dermatitis lesion was greatly reduced in its severity. On the 12th day there was some thickening of the skin and increased scaliness of the surface, but from then onwards the lesion regressed, and no evidence of its presence could be seen after the end of the fourth week.

It has already been mentioned that in Area 4, which had been treated with croton oil, the dermatitis lesion was more advanced than in Area 1 at the 8th day after inoculation. From then onwards the successive stages of the lesions in Areas 1 and 4 were reached at about the same times, but the lesion in Area 4 was more severe in that it was more confluent, and showed greater thickening of the skin and more exuberant outgrowths.

Histology.—Fig. 5 to 8 show the histological features of the four areas of skin in Rabbit 7, 20 days after inoculation with dermatitis virus. The section from Area 1 (Fig. 5) shows part of a discrete dermatitis lesion on normal skin. The lesion is essentially an epithelial hyperplasia involving mainly the upper parts of the hair follicles, and at this stage is mostly below the level of the epidermis, so that it can be readily differentiated from the Shope papilloma. A comparison of Fig. 5 with Fig. 6, 7 and 8 will show that similar modifications have been produced on dermatitis virus infection as have been shown in Expt. 1 with Shope papilloma virus infection, in that subsequent inoculation with papilloma virus (Fig. 6) and pre-treatment with the carcinogenic agent (Fig. 7) have had a suppressing effect, while croton oil has had an intensifying action (Fig. 8). It is also evident that, as with papilloma infection, the carcinogenic agent has had a greater suppressing effect than subsequent inoculation with the other virus. These effects, however, have all been much more intensive on the dermatitis infection. The early stage of papilloma infection in Fig. 6 is somewhat less well developed than it would be on normal skin.

These differences in the dermatitis lesions are still evident at 35 days in sections from Rabbit 5, where there is considerable hyperplasia of the hair sheaths and epidermis in Area 1, little evidence of dermatitis infection in Area 2, almost complete healing in Area 3, and much greater hyperplasia in Area 4 than in Area 1. A later series of sections from Rabbit 7 at 56 days shows advanced healing

of the dermatitis lesion in Areas 1 and 4, and no evidence of its presence in Areas 2 and 3. The papillomas in Area 2, as shown by sections up to 41 days, are smaller than those arising from normal skin in the absence of dermatitis infection as in Area 1 of Expt. 1. In later sections, however, this difference in size is not noticeable, but the papillomas all tend to show the effects of the associated dermatitis infection in that they have the filiform appearance already described in the papillomas from Area 2, Expt. 1.

Experiment 3 : *mixed papilloma and dermatitis infection.*

In Exp. 3, Areas 1, 2 and 4 of the skin of Rabbits 9 to 12 were inoculated with a mixture of Shope papilloma virus and sheep dermatitis virus on December 15, 1944. The further details of the treatment of Areas 2, 3 and 4 are shown in Table III. The first evidence of infection was noted on the 8th day in all three

TABLE III.—*Expt.* 3 (*Rabbits* 9 *to* 12) : *Virus Inoculations and Chemical Pre-treatment.*

Skin area.	Pre-treatment.	Virus inoculations.
1	None	Mixed Shope papilloma and sheep dermatitis.
2	9 : 10-Dimethyl-1 : 2-benzanthracene	,, ,, ,,
3	,, ,,	None.
4	Croton oil	Mixed Shope papilloma and sheep dermatitis.

inoculated areas and took the form of the characteristic erythema and scaliness of the first stage of dermatitis infection. In the previously untreated skin of Area 1 the severity of the dermatitis lesion was greatly reduced by the presence of the papilloma infection, and regression was apparently complete by the 25th day in Rabbits 9, 10 and 11 and by the 45th day in Rabbit 12. The effects on the dermatitis lesion of pre-treatment with the carcinogenic agent and with croton oil were similar to those found in Expt. 2 in that the lesion was less severe in Area 2 and intensified in Area 4, but regression of the dermatitis component

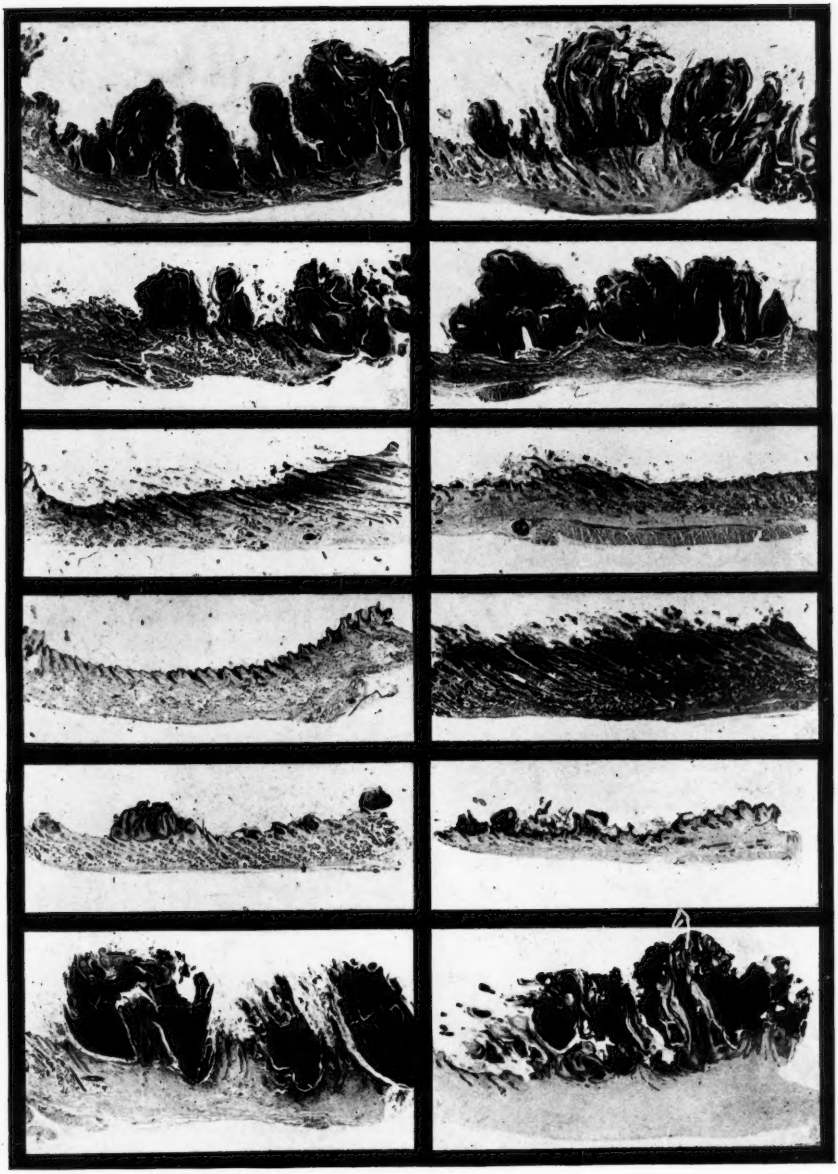
DESCRIPTION OF PLATE.

FIGS. 1–4.—Sections from Rabbit 2, Expt. 1, 34 days after inoculation of Shope papilloma virus. H. and E. × 4. Fig. 1.—Area 1, inoculated with papilloma virus only. Fig. 2.—Area 2, inoculated with dermatitis virus 14 days after inoculation of papilloma virus. Fig. 3.—Area 3, treated with 9 : 10-dimethyl-1 : 2-benzanthracene before inoculation of papilloma virus. Fig. 4.—Area 4, treated with croton oil before inoculation of papilloma virus.

FIG. 5–8.—Sections from Rabbit 7, Expt. 2, 20 days after inoculation of sheep dermatitis virus. H. and E. × 4. Fig. 5.—Area 1, inoculated with dermatitis virus only. Fig. 6.—Area 2, inoculated with Shope papilloma virus 6 days after inoculation of dermatitis virus. Fig. 7.—Area 3, treated with 9 : 10-dimethyl-1 : 2-benzanthracene before inoculation of dermatitis virus. Fig. 8.—Area 4, treated with croton oil before inoculation of dermatitis virus.

FIG. 9–11.—Sections from Rabbit 10, Expt. 3, 20 days after inoculation with a mixture of Shope papilloma virus and sheep dermatitis virus. H. and E. × 4. Fig. 9.—Area 1, mixture of papilloma and dermatitis viruses inoculated on untreated skin. Fig. 10.—Area 2, treated with 9 : 10-dimethyl-1 : 2-benzanthracene before inoculation of virus mixture. Fig. 11.—Area 4, treated with croton oil before inoculation of virus mixture.

FIG. 12.—Section from Area 4, Rabbit 12, Expt. 3, 49 days after inoculation with a mixture of Shope papilloma virus and sheep dermatitis virus, pre-treatment with croton oil. H. and E. × 4.



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of the combined lesion appeared to occur at about the same time in all three inoculated areas.

Papillomas could be seen in Areas 1 and 2 of all four rabbits at the 18th day, but in Area 4 they could not be detected in the thick nodular growth of the dermatitis infection until the 25th day. In their later development the papillomas in Area 1 were somewhat smaller than they would have been in the absence of dermatitis infection as in Area 1 of Expt. 1, and were about equal in size to those in Area 2 of Expt. 1, where the inoculation of papilloma virus was followed 14 days later by the inoculation of dermatitis virus. In the two other inoculated areas the number of papillomas was similar to that in Area 1, but the effects of chemical pre-treatment were seen in the smaller size of the papillomas in Area 2 following treatment with the carcinogenic agent, and in the larger size and greater degree of keratinization of the papillomas in Area 4 following treatment with croton oil. These differences in the papillomas could be detected in Area 2 up to 7 to 9 weeks, and in Area 4 for as long as 10 months.

Histology.—Fig. 9 to 11 show the histological appearances of the three inoculated areas of skin in Rabbit 10, 20 days after inoculation. In the previously untreated skin of Area 1 (Fig. 9) the papillomas are slightly smaller than they would be in the absence of dermatitis infection, while there is little evidence of the dermatitis component of the mixed infection. Pre-treatment with the carcinogenic agent in Area 2 has caused a further retardation in the development of the papillomas with early complete regression of dermatitis (Fig. 10). On the other hand, the croton oil pre-treatment in Area 4 has intensified the dermatitis component of the mixed infection to such an extent that the papillomatous component is totally obscured (Fig. 11). This lesion has an appearance identical to the later pseudopapillomatous stage of dermatitis infection in normal rabbit skin with the characteristic pockets of pus in enlarged hair follicles. A section from Area 3 shows that the hyperplasia of the epidermis and hair sheaths and the mononuclear infiltration of the dermis produced by the carcinogenic agent alone are still present 3 weeks after the applications have terminated.

In sections from Areas 1 and 2 of Rabbit 11 at 35 days and Rabbit 12 at 49 days there is no evidence of dermatitis infection and the papillomas are of similar size, but they are rather smaller and more elongated and keratinized than in Area 1 of Expt. 1. In Area 4, however, it can be seen that papillomas of a somewhat irregular shape are developing from the epithelium of the outgrowths of the dermatitis lesion (Fig. 12). In later sections, and especially in those from Area 4, the papillomas still retain their elongated and more keratinized appearance.

Infectivity Tests.

On October 3, 1945, ten months after the commencement of these experiments, the surviving rabbits were No. 1 and 3 of Expt. 1, No. 5, 6, 7 and 8 of Expt. 2, and No. 12 of Expt. 4, all bearing papillomas on the areas of skin that had been inoculated with papilloma virus. Rabbits 3, 6 and 12, one from each experiment, were killed, and a portion of each of the papillomatous lesions, except that from Area 1, Rabbit 3, was ground with sand and suspended in saline to make a 10 per cent extract. The 7 extracts were then inoculated by scarification on 7 separate areas of the skin of each of two rabbits. The first result of this test was seen at 12 to 14 days in the appearance of discrete dermatitis lesions in

both rabbits in the areas of skin inoculated with extracts from Rabbit 12. These dermatitis lesions had almost completely regressed 5 weeks after inoculation, but at a further examination 14 days later a crop of about 10 papillomas was observed in both rabbits on the areas of skin inoculated with the extract prepared from Area 4 of Rabbit 12, and these papillomas appeared to have developed on the sites previously occupied by the dermatitis lesions.

A second series of infectivity tests was made on November 7, 1945, on papillomas removed by operation from Area 2, Rabbit 1, Expt. 1, and from Area 2 of Rabbits 5, 7 and 8, Expt. 2. Extracts of these papillomas were inoculated in duplicate on the skin of two rabbits. A confirmatory test was also made on December 19, 1945, on papillomas preserved in glycerol from Areas 1 and 4 of Rabbit 12, Expt. 3. The extracts of these glycerolated lesions were tested on one rabbit only, but on areas of skin twice as large as those used in the previous tests.

TABLE IV.—*Infectivity of Papillomas from Expt.* 1, 2 *and* 3, 10–11 *Months after Inoculation.*

Description of test material.				Date of test.	Result of test. Number of lesions per inoculation area of skin.	
Expt.	Date of inoculation of papilloma virus.	Area.	Inoculation of dermatitis virus.	Rabbit number.		Papilloma.	Dermatitis.
1	1.xii.44	2	14 days later	1	7.xi.45	0	0
				3	3.x.45	0	0
		3	None	3	3.x.45	0	0
		4	„	3	3.x.45	0	0
2	21.xii.44	2	6 days earlier	5	7.xi.45	0	0
				6	3.x.45	0	0
				7	7.xi.45	0	0
				8	7.xi.45	0	0
3	15.xii.44	1	Simultaneous	12	3.x.45	0	2
					19.xii.45*	2	2
		2	„	12	3.x.45	0	1
		4	„	12	3.x.45	10	10
					19.xii.45*	3	>30

* Second test with papillomas stored in 50 per cent glycerol.

It will be seen from Table IV, where the results of the infectivity tests are arranged in accordance with the origin of the test material, that none of the papilloma extracts from Expts. 1 and 2 proved infective, whereas both dermatitis and papilloma infections were obtained from the papillomas of Expt. 3. It will also be noted in Expt. 3 that, in the test of October 3, papillomas were obtained only from Area 4 of Expt. 3, but this would appear to be of little significance, since the glycerolated material from both Areas 1 and 4 yielded papillomas in about equal numbers in the further test of December 19. The results, therefore, show that papillomas that had arisen from skin infected simultaneously with dermatitis virus contained both papilloma virus and dermatitis virus in a virulent form, whereas the presence of neither virus could be demonstrated in any of the other papillomas.

DISCUSSION.

The mutual effects of double infection and of chemical pre-treatment of the skin on Shope papilloma and sheep dermatitis infections in the rabbit are summarized in Table V. It will be seen that, in general, the inoculation of the two viruses on the same area of skin has caused a reduction in the severity of both

virus infections. When the two viruses are inoculated at different times the suppressing effect is greater on the second virus infection than on the first, but when the two viruses are inoculated simultaneously on normal skin the dermatitis infection is almost completely suppressed, while the papilloma infection is little affected. These results would therefore appear to offer a new example of the now well-known phenomenon of virus interference which has recently been

TABLE V.—*Effect on Shope Papilloma and Sheep Dermatitis Infections of Double Infection and Chemical Pre-treatment of the Inoculated Skin.*

Treatment.	Effect on virus infection.		Recovery of active virus at 10–11 months.	
	Papilloma.	Dermatitis.	Papilloma.	Dermatitis.
Preceded by the other virus	Reduced	Reduced	−	−
Mixed with the other virus	Slightly reduced	Greatly reduced	+	+
Followed by the other virus	,, ,,	Slightly reduced	−	−
Preceded by croton oil	Slightly intensified	Intensified	−	−
Preceded by 9 : 10-dimethyl-1 : 2-benzanthracene	Reduced	Greatly reduced	−	−

reviewed by Andrewes (1944). The results of the infectivity tests are also summarized in Table V, and are of considerable interest because they would appear to afford a clue to the mechanism of interference between these two virus infections. The successful recovery of dermatitis virus from papillomas that have arisen from skin simultaneously infected with dermatitis virus, but not from papillomas associated with previous or later dermatitis infection, leads to the following interpretation:

When the normal rabbit epithelium is inoculated successively with the two viruses, the first virus infects a proportion of the normal cells to which it prevents the entry of the second virus, so that the latter is limited in its attack to normal cells not yet occupied by the first virus. At the same time the second virus, by a similar blockading action, prevents new cells being attacked by the first virus infection. The result is that both infections have less available territory, but since the first virus has had more time in which to consolidate its position, the suppressing effect is greater on the second virus infection. The two infections thus develop side by side, but without interchange of virus, so that each virus is segregated to the appropriate cells. The consequence is that when the dermatitis lesion regresses, the papillomas progress as an independent infection and do not provide a source of dermatitis virus.

On the other hand, the late recovery of dermatitis virus from papillomas when the rabbit skin is inoculated with a mixture of the two viruses shows that both viruses gain entry into a certain proportion of epithelial cells, so that it must be presumed that it requires some time for one virus to blockade the entry of the other. The immediate development of these doubly-infected cells is at present in doubt, but the recovery of dermatitis virus from papillomas 10 months later shows that a certain number ultimately form papillomas. That the doubly-infected cells may initially take on the form of a dermatitis lesion is perhaps indicated in Figs. 11 and 12, where it is shown that outgrowths characteristic of dermatitis infection are developing into Shope papillomas. In any case it is evident that once the dermatitis virus gains entry into the cells of a papilloma, it becomes a passenger and remains latent in that it is apparently non-pathogenic

to the carrier cells, but at the same time retains its pathogenicity for the normal rabbit skin.

The results that have been obtained with the papilloma virus in these experiments are more difficult to explain. The failure to find active virus in the papillomas of Expt. 1 and 2 is to be expected because extracts of papillomas growing in domestic rabbits are rarely infective, although the presence of virus can readily be demonstrated by serological methods (Shope, 1935; Kidd, Beard and Rous, 1936). On the other hand, the papillomas derived from the simultaneous inoculation of papilloma and dermatitis virus in Rabbit 12 have proved infective, and the yield of papillomas has been greater than that usually obtained on the rare occasions on which domestic rabbit papillomas can be transmitted, when only one or two papillomas appear on the inoculated skin. It would therefore appear that the successful outcome of the infectivity tests for papilloma virus in this experiment is in some way due to the associated infection of the papilloma cells with dermatitis virus. It may be that the dermatitis virus has so altered the metabolism of the papilloma cells that it has made available the conditions necessary for the maintenance of papilloma virus in a virulent state. On the other hand, it is possible that the dermatitis virus in the infective extracts of these papillomas may, on inoculation to normal rabbits, have so altered the epithelial cells that they have become susceptible to infection by the otherwise ineffective papilloma virus.

The evidence provided by the infectivity tests has thus shown that interference between the virus infections of Shope papilloma and sheep dermatitis can take place in two ways. The mode of interference which occurs when the two viruses are inoculated at different times is similar to that between two bacteriophages attacking the same bacterium, first reported as phage blockage by White (1937) and later described by Delbrück (1945) as mutual exclusion, whereby the invasion of a bacterium by one bacteriophage prevents the entry of a second. Many other examples of this type of interference in animal and plant viruses have been described and are reviewed by Findlay and MacCallum (1937), Price (1940) and Andrewes (1944). It was at first thought that virus interference occurred only between related strains of viruses, but the more modern view expressed by Ziegler, Lavin and Horsfall (1944) and Delbrück (1945) that this is not a necessary condition is supported by the work presented here.

The second mode of virus interference described here, where the host cells are infected by both viruses of which one, the papilloma virus, determines the nature of the lesion, has not been observed in bacteriophage studies, but is probably of a similar nature to that reported by Andrewes (1940) in dual infection by Virus III and fibroma virus in rabbits, where Virus III either totally suppresses the other virus or forms inclusion bodies in the cells of the fibromatous lesions. Other instances of dual virus infection have been reported, and the formation of nuclear inclusions of one virus infection and cytoplasmic inclusions of another virus infection has been demonstrated in single cells of the chick embryo by Anderson (1942). Shope papillomas can also be readily superinfected by other viruses with the production of characteristic cytological changes, as has been shown by Levaditi and Schoen (1936) and Syverton and Berry (1936, 1938). The virus superinfection described here, however, differs from those previously reported in that the dermatitis virus, probably because of its relatively low virulence, produces an apparently silent infection in the Shope papilloma

and, in some manner yet to be determined, enables the demonstration of active papilloma virus. This enhancement of the activity of the usually avirulent papilloma virus in domestic rabbits will be of considerable value in mammalian tumour virus studies if it should prove to afford a method of establishing a domestic rabbit strain of Shope papilloma that readily provides infective extracts. It may also be of more general value in giving a lead to methods whereby other latent or cryptic viruses may be stimulated into activity.

The results of pre-treatment of the rabbit skin with the carcinogenic agent, 9 : 10-dimethyl-1 : 2-benzanthracene, and croton oil are of considerable interest in that two agents that cause hyperplasia of the epidermis have, on the one hand, inhibited and, on the other, stimulated the development of both virus infections. It has been shown by Berenblum (1941) and Mottram (1944) that croton oil, used in a similar manner, has a stimulating effect on the production of skin tumours in mice by carcinogenic agents, but hitherto there has been no report of its stimulating action on the evolution of dermotropic virus infections, such as has been shown here. Other hyperplastic reagents that have been shown to render rabbit skin more susceptible to Shope papilloma virus infection are tar, methylcholanthrene, benzpyrene, and a mixture of turpentine and acetone (Friedewald, 1942).

The stimulating effect on Shope papilloma obtained by Friedewald (1942) with carcinogenic hydrocarbons is the opposite to that described here with 9 : 10-dimethyl-1 : 2-benzanthracene, but this difference is probably due to differences in the conditions of the experiments. In the first place, 9 : 10-dimethyl-1 : 2-benzanthracene is a more potent carcinogenic agent in the rabbit than methylcholanthrene or benzpyrene (Berenblum, 1945), and in the present experiments the applications of this agent were just sufficient to produce temporary warts in 3 of the 12 rabbits, so that the papilloma virus was inoculated at a time when the carcinogenic process was well advanced. In Friedewald's experiments, on the other hand, the hydrocarbons were applied 4 to 6 times within a period of only two weeks, so that, at the time of the inoculation of papilloma virus, the rabbit skin was probably in an initial hyperplastic phase of the carcinogenic process, and in a state more akin to that produced by croton oil. This question, however, requires further investigation, particularly in view of the possibility that, in the present experiments, the inoculation of virus may have been impeded to some extent by the growth of the fur during the preliminary treatment with the carcinogenic agent. This interference may have lessened the crop of papillomas in Expt. 1, but it does not appear probable that it could have retarded the growth of the individual papillomas.

In any case it is of interest that the continued application of a carcinogenic agent just short of tumour formation has produced an inhibiting effect on two dermotropic virus infections similar to that of virus interference, whereas a non-carcinogenic hyperplastic reagent, croton oil, has had a stimulating action. It is possible that the inhibiting effects are related in the sense that the carcinogenic agent causes an alteration in the metabolism of the epithelial cells similar to that produced by a virus, and indeed the ultimate phase in carcinogenesis may be associated with the activity of a normally latent virus. In support of the latter view is the great similarity between the Shope papilloma and chemically-induced skin tumours of the rabbit (Rous and Kidd, 1939), the finding of McIntosh (1933) and McIntosh and Selbie (1939) that virus agents can be obtained from

chemically-induced sarcomas in the fowl, and the demonstration in mouse mammary cancer of a virus which is greatly modified in its activity by the hereditary constitution and hormonal stimulation of the mammary tissue of the host (Bittner, 1941 ; Dmochowski and Gye, 1944 ; Dmochowski, 1944).

SUMMARY.

When Shope papilloma virus and sheep dermatitis virus are inoculated at different times in the same area of skin in the rabbit, both infections are reduced in severity, the suppressing effect on either infection being greater when it is preceded by the other.

When the two viruses are inoculated simultaneously the dermatitis virus infection is almost completely suppressed, while the papilloma virus infection is little affected and produces papillomas which contain both viruses in a virulent form up to 10 months after inoculation.

Prolonged pre-treatment of the inoculated skin with the carcinogenic agent, 9 : 10-dimethyl-1 : 2-benzanthracene, has an inhibiting effect on both virus infections, whereas a short pre-treatment with croton oil has a stimulating effect.

The findings are discussed with reference to the virus tumours and the phenomena of virus interference and superinfection.

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THE EFFECT OF FEEDING CARCINOGENIC HYDROCARBONS DISSOLVED IN AQUEOUS SOAP SOLUTION ON THE STOMACH OF CBA MICE.

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THE aim of this investigation was to observe the effect of aqueous solutions of carcinogenic hydrocarbons on the stomachs of CBA mice. Aqueous solutions mix better with the contents and secretions of the stomach than fatty solutions, and come, therefore, into better contact with the lining epithelium. This is especially true for the stomach ; below the pylorus the emulsifying action of bile facilitates contact between lipids and lipid-soluble substances and the mucous membrane. By exhibiting carcinogens in soap solution it was hoped to induce adenocarcinoma of the stomach. This hope has not yet been fulfilled.

EXPERIMENTS.

The preparation of aqueous soap solution of carcinogenic hydrocarbons has been published (Beck, 1943). The hydrocarbon is dissolved in ethereal soap solution, water is added and the ether evaporated by heating. Solutions of 0·01 per cent 3 : 4-benzpyrene and 0·0025 per cent 1 : 2 : 5 : 6-dibenzanthracene were prepared. These solutions were substituted for drinking water at the rate of 0·04 mg. per mouse per day for benzpyrene, and 0·01 mg. for dibenzanthracene.

Fourteen CBA mice received benzpyrene up to 97 weeks and 16 CBA mice dibenzanthracene up to 71 weeks, and the latter subsequently received 0·1 per cent croton oil in soap solution up to 90 weeks.

RESULTS.

Six mice of those treated with benzpyrene survived the 42nd week, in which the first papilloma of the forestomach was discovered *post mortem*. All of these six mice (Nos. 4031–4036) showed tumours of the forestomach, two papillomas after 42 and 53 weeks respectively and four squamous carcinomas after 69, 77, 93 and 97 weeks. (Fig. 1–3.)

Ten mice of the dibenzanthracene group survived the 72nd week, and two had branching papillomas after the 72nd and 94th week respectively.

DISCUSSION.

The literature about cancer of the stomach in mice and rats is reviewed by Klein and Palmer (1941), Sugiura (1942) and Kirby (1943). Positive results with carcinogenic hydrocarbons were claimed by the following investigators :

Oberling, Sannié, Guérin and Guérin (1936) and Waterman (1936) induced

* Working under a full-time grant from the British Empire Cancer Campaign.

independently malignant tumours of the fore stomach byfeeding mice with benzpyrene in lard. The latter failed to produce tumours with a colloidal solution.

Van Prohaska, Brunshwig and Wilson (1939) observed papillomas in two mice fed with 1·0 per cent methylcholanthrene.

Lorenz and Stewart (1940) obtained squamous carcinoma in ten mice of Strain A fed with an aqueous emulsion of olive or mineral oil solutions containing methylcholanthrene, but failed to induce tumours in Strains C3H, C57 Black and C57 Brown. Dibenzanthracene gave negative results in all four strains.

Collins, Gardner and Strong (1943) fed mice of Strains C3H, A, NH, ChI and C57 with 0·6 mg. benzpyrene in sesame oil per week and obtained papillomas and squamous carcinomas of the forestomach. One carcinoma was successfully transplanted.

Strong, Collins and Durand (1943) have described adenocarcinoma of the glandular stomach in NH mice, after subcutaneous injections of 1 mg. methylcholanthrene. Such selected mice they termed NHO.

Peacock and Kirby (1944) produced in the forestomach papillomas with a tendency to malignant growth by feeding stock mice with benzpyrene and methylcholanthrene in olive oil or cod liver oil. They stressed the differences between gastro-papillomatosis associated with dietary deficiencies and papillomas induced by carcinogenic hydrocarbons.

Strong (1945) injected subcutaneously mice of Strain NH with 1 mg. methylcholanthrene for eight generations, and selected the treated mice for resistance towards local tumour induction. The earlier generations of the strain, now named NHO, showed a relatively high incidence of induced adenocarcinomas of the stomach ; the later generations developed similar adenocarcinomas spontaneously.

Kirby, of this Department, in an unpublished experiment fed 13 Wistar rats with benzpyrene in soap solution for 82 weeks and 12 rats of the same strain with methylcholanthrene in soap solution for 61 weeks, at the rate of 6 mg. per week. The writer followed up the experiment by giving 0·1 per cent croton oil in soap solution, until the 10 surviving rats died, from 5 to 29 weeks later. No tumour was observed in the stomachs of these 25 rats.

Berenblum (1941) first demonstrated the co-carcinogenic action of croton oil and croton resin.

Subsequently Mottram (1944*a*, *b*) showed that by painting the skin of mice with either croton oil or croton resin, before or after a single local application of benzpyrene, the incidence of skin tumours was increased.

The papillomas induced by dibenzanthracene seem to be the first to be recorded. Reinhard and Candee (1936), Branch (1936) and Lorenz and Stewart (1940) noted negative results for mice. Sugiura notes positive results for dibenzanthracene referring to the paper of Van Prohaska, Brunshwig and Wilson (1939), but according to their original paper these investigators obtained their results with methylcholanthrene.

As already stated above, two cases of papilloma of the forestomach occurred in the present series in mice which received dibenzanthracene followed by croton oil.

That the glandular mucosa of the stomach can respond to carcinogenic hydrocarbons was shown by Stewart and Lorenz (1941), who injected methylcholanthrene into the wall of the glandular stomach and obtained adenocarcinoma in Strain A mice ; and by Strong (1945), who obtained adenocarcinoma of the

Fig. 1.

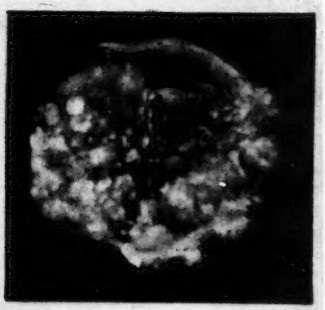

Fig. 2.

Fig. 3.

FIG. 1.—Peritoneal aspect of stomach of Mouse No. 4034. (Twice natural size.)

FIG. 2.—Internal aspect of the same stomach.

FIG. 3.—Junction of an area of hyperkeratosis of the forestomach of Mouse No. 4036 with keratinizing squamous carcinoma. H.E. × 260.

stomach in NH mice by subcutaneous injections of methylcholanthrene. Resistance of the mucosa to ingested carcinogens can be explained in several ways.

Mucus may act as a barrier which prevents the carcinogens from reaching the secreting cells. Not only is mucus relatively difficult to mix with watery solutions, but the actual flow may have a protective effect quite apart from the physical properties of mucus. An example of such protective action is the flow of urine which can prevent ascending infection in ureters transplanted into the large bowel.

Alternatively it may be that contact between a carcinogen and a resting cell is not sufficient to induce neoplasia; some degree of proliferative activity may be necessary to allow entry of the carcinogen into the nucleus during mitosis.

One must also consider the possibility that glandular epithelium is essentially less susceptible than squamous epithelium to these particular carcinogens.

SUMMARY.

1. CBA mice were fed with an aqueous soap solution of benzpyrene (0·04 mg. per day) for periods of 3 to 97 weeks, and dibenzanthracene (0·01 mg. per day) for periods of 20 to 71 weeks, followed by 0·1 per cent croton oil in soap solution up to 90 weeks.

2. No tumours occurred in the glandular stomach.

3. Every one of the 6 mice surviving for 42 weeks showed tumours of the forestomach (2 papillomas and 4 carcinomas) in the benzpyrene group.

4. Two papillomas were observed among 10 mice surviving 72 weeks in the dibenzanthracene group.

5. The reasons for the lack of susceptibility of the glandular stomach to ingested carcinogens are discussed.

I wish to thank Dr. Peacock for examining the sections.

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RENAL EFFECTS OF PROTEIN-DEFICIENT VEGETABLE DIETS: A FUNCTIONAL AND HISTOLOGICAL STUDY.

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IT is not clear from clinical reports whether oedema of protein deficiency (oedema of malnutrition) is accompanied by impairment of renal function. It seemed of interest, therefore, to study the kidney of animals in which oedema had been produced experimentally by nutritional measures. The dietary regime which leads to the occurrence of oedema of malnutrition in human beings is in most instances a low protein vegetable diet of low calorific value. Both the war of 1939–45 (Perakis and Bakalos, 1943; Holmes, 1944; Stevenson, 1944; Mollison, 1946) and that of 1914–18 (Maver, 1920) furnish numerous examples of cases of oedema due to such diets. Diets of this type have actually been used by Denton and Kohman (1918), Kohman (1920), Maver (1920) and Frisch, Mendel and Peters (1929) to produce experimental oedema in rats. Similar diets were therefore chosen for this investigation.

METHODS.

Experimental Animals.

Adult male albino rats ranging in weight from 140 g. to 180 g. were used.

Diets.

The standard diet (ST) was as follows: casein, 18 per cent; wheaten starch, 29·5 per cent; hardened ground nut oil, 9 per cent; dried yeast, 11 per cent; salt mixture ('U.S. Pharmacopeia IX,' revised 1937, Vitamin A and D test diet, salt mixture No. 2), 3 per cent; cod liver oil, 2 per cent; water, 27·5 per cent; dl-Tocopheryl acetate (Ephynal "Roche"), a sufficient amount. A hundred grammes of this diet provide approximately 320 calories. Each animal received about 20 g. per 24 hours.

Protein deficient diet No. 1 (Carrot diet = CT) was modelled on that of Kohman (1920) and of Frisch, Mendel and Peters (1929), who showed that such a diet produced oedema and hypoproteinaemia in rats. It had the following composition: fresh carrots, 85·5 per cent; wheaten starch, 7 per cent; hardened ground nut oil, 1·2 per cent; salt mixture, 4·2 per cent; cod liver oil, 2·1 per cent; a sufficient amount of vitamin B (Messrs. Roche Products' "Benerva" tablets containing aneurin, riboflavin and nicotinic acid) and tocopheryl acetate.

Protein-deficient diet No. 2 (Turnip diet = TT) had the same composition as CT with fresh turnips taking the place of fresh carrots.

The supply of CT or TT to the rats was not restricted, but it was found that

the animals ate approximately 50 g. of these diets per 24 hours, which represented an intake of approximately 35–40 calories. It should be noted that carrots contain approximately 0·7 per cent, and turnips approximately 0·8 per cent of protein.

The Medical Research Council War Memorandum No. 14 was used for the calculation of calorific values.

Determinations of Water Content.

Samples of heparin plasma or tissues were dried at 105° C. until of constant weight.

Estimation of Plasma Proteins.

A micro-Kjeldahl method employing direct Nesslerization and correction for non-protein nitrogen was used (Hawk and Bergeim, 1942).

Renal Function Tests.

The details of the experimental procedures for the determination of simultaneous inulin and diodone clearances in rats have been described in a previous paper (Dicker and Heller, 1945). Inulin in plasma and urine was determined by the method of Smith, Goldring and Chasis (1938). Diodone iodine in plasma and urine was determined by Alpert's (1941) method. Inulin (Kerfoot & Co.) and Per-Abrodil (Bayer Products, Ltd.) were used. Chloride in plasma was estimated by Whitehorn's (1921) method, and chloride in urine by that of Volhard-Arnold. Chloride was expressed as NaCl. Definition and method of calculation of inulin clearance ($= C_{IN}$ = glomerular filtration rate = GFR), diodone clearance at low plasma diodone levels ($= C_D$ = effective renal plasma flow = RPF) and total tubular excretory mass (Tm_D) conform with those outlined in the above-mentioned paper (Dicker and Heller, 1945), with the exception that the factors F (= plasma diodone iodine/ultrafiltrate diodone iodine ratio) and $W \left(= 1{\cdot}00 - \frac{\text{per cent plasma solids}}{100}\right)$ used in the calculation of Tm_D were newly determined for the plasma of protein-deficient rats. The mean of 15 determinations of W was $0{\cdot}94 \pm 0{\cdot}002$ as against $0{\cdot}91 \pm 0{\cdot}002$ in normal animals. F, as determined by the ultrafiltration of the plasma of 9 hypoproteinaemic rats, was 0·9 (F in normal rats = 0·7). The rate of tubular reabsorption of chloride (T_{cl}) was calculated as follows:

$T_{cl} = (P_{cl} \times C_{IN}) - (U_{cl} \times V)$, where P_{cl} = concentration of plasma chloride in mg./100 ml., C_{IN} = inulin clearance in ml./100 g./min., U_{cl} = concentration of urinary chloride in mg./100 ml., and V = urine flow in ml./100 g./min. In order to permit the comparison of rates of tubular water reabsorption (T_w = GFR − V) and rates of tubular reabsorption of chloride (T_{cl}) at different rates of glomerular filtration, T_w and T_{cl} were expressed as the percentages of water and chloride filtered.

The ability of the kidneys of rats on protein-deficient diets to dilute and to concentrate the urine was tested in the following manner:—Dilution test: rats which had been deprived of food for 6 hours received 5 per cent of their body weight of tap water by stomach tube, and were put singly into glass metabolism cages. Urine samples were collected at 30-minute intervals for a period of 3 hours. The volume of the urine samples was measured to estimate the

diuretic response, and the specific gravity of each sample was determined. Concentration test: the rats were deprived of food and water for 24 hours and the specific gravity of samples collected after 8 and 24 hours was measured. Heller's (1941) apparatus was used for these measurements. Special urinometers were used which permitted estimations of the specific gravity to the fourth decimal. It should be pointed out that an interval of at least 24 hours was allowed between the dilution and the concentration tests.

Post-mortem Examination.

Autopsy was performed immediately after death. Any ascitic fluid was removed, measured and analysed for its protein content. The liver and kidneys were removed and fixed in 10 per cent formol-saline, blocks being cut for histological study.

Histological Methods.

Frozen and paraffin sections were cut of liver and kidney and stained with Scharlach R, haematoxylin and eosin, Masson's trichrome stain, and Kossa's stain for calcium.

Statistical Treatment of Results.

Fisher's " t " and correlation coefficients (" r ") were calculated according to Mainland (1938), and Fisher and Yates' (1943) tables were consulted. Small sample methods were used for populations numbering less than 20.

RESULTS.

Before feeding with a protein-deficient vegetable diet the animals were kept on the standard diet (ST) for about 14 days, the body weight showing a slow, steady increase during this preparatory period. When a protein-deficient diet (CT or TT) was substituted for the standard diet the body weight of the rats fell steadily and on the whole consistently. The mean loss of body weight of 112 adult protein-deficient rats amounted to $35{\cdot}4 \pm 1{\cdot}85$ (S.E.) per cent in about 36 days. The last weighings were made on the day of the final renal function tests. It was noticed that the body weight of adult protein-deficient rats showed, occasionally, paradoxical increases during the last week of observation. For instance, rat No. 16 on TT diet since May 8 was found to weigh 133 g. on June 8, but 143 g. on June 11, decreasing again to 127 g. on June 13. Such rats were subsequently found to suffer from ascites.

External Appearance of Rats on Protein-deficient Vegetable Diets.

Towards the end of the period of observation the rats became emaciated and their coats lost the normal smooth appearance. However, there was a general absence of intercurrent or secondary infection and no ophthalmia. The skin of the tail and feet was smooth. The lack of spontaneous movements was very noticeable. No diarrhoea was observed in these animals: the faeces were well formed but pale. Death occurred occasionally, without previous spasticity or convulsions, in animals which had given no outward signs of sudden deterioration.

Level of Plasma Proteins.

Blood for the determination of plasma proteins was collected at the same time as that for renal clearance estimations. The mean value for plasma proteins in the rats on carrot diet was 4·67 ± 0·120 g./100 ml. (S.E. of mean of 42 observations); the mean value in the 31 rats on turnip diet was 4·26 ± 0·097 g./100 ml. A series of plasma protein determinations in 23 normal adult animals gave a mean of 7·58 ± 0·682 g./100 ml., showing that feeding with CT or TT lowered the plasma protein concentration by approximately 44 per cent.

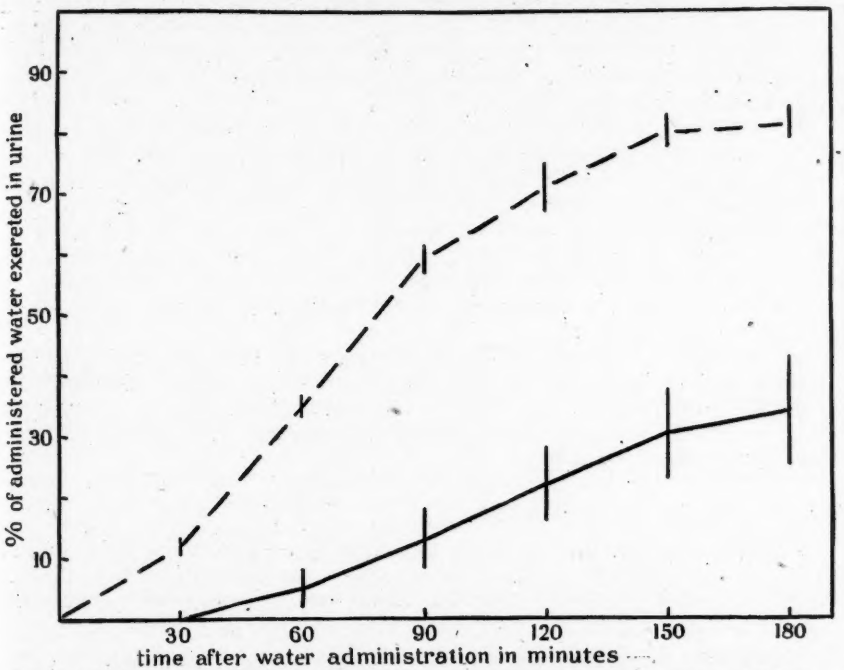

FIG. 1.—Water diuresis in protein-deficient rats on "turnip diet." ——— protein-deficient animals. – – – – same animals before institution of protein-deficient turnip diet. All animals received 5 per cent of their body weight of water by stomach tube. The vertical lines indicate the standard error.

Water Diuresis in Rats on Protein-deficient Vegetable Diets.

In the course of experiments designed to test the ability of the kidneys of protein-deficient rats on TT to dilute the urine, which necessitated the administration of 5 per cent of their body weight of water by stomach tube, it was observed that the urinary excretion of the administered water was delayed in many cases (Fig. 1). The total amount of extra water excreted in 3 hours was also decreased: 34·2 ± 9·22 per cent (21 observations) of the administered water in the protein-deficient animals, as compared with 81·5 ± 2·66 per cent (21) in the same animals before feeding with a protein-deficient diet started.

Renal Function Tests in Rats on Protein-deficient Vegetable Diets.

A. *Dilution and concentration tests.*

Two series of dilution and concentration tests were performed, one during the period when the animals were on standard diet, and a second towards the end of the period of feeding with a protein-deficient diet. An interval of at least 24 hours was interposed between the dilution and the concentration test. The maximum dilution obtained in 20 rats on standard diet was $1{\cdot}005 \pm 0{\cdot}0005$. The same rats after several weeks of protein-deficient diet showed a maximum dilution of $1{\cdot}010 \pm 0{\cdot}0016$. The difference between these figures is highly significant ($t = 4{\cdot}486$, $P < 0{\cdot}001$). Rats on both protein-deficient diets were used for these experiments. The results were pooled, since responses of the same magnitude were obtained in both series.

Concentration tests on the same group of animals performed some days before and several weeks after the institution of protein-deficient feeding gave the following mean results for urines collected between the 8th and the 24th hour of water deprivation: specific gravity $= 1{\cdot}070 \pm 0{\cdot}0037$ for animals on ST, and $1{\cdot}041 \pm 0{\cdot}0044$ for the same animals on CT or TT ($t = 5{\cdot}087$, $P < 0{\cdot}001$). The inability of the protein-deficient animals to concentrate to the same degree as during the period of standard feeding was, at least in some cases, unlikely to have been entirely due to changes of tubular function. This may be illustrated by the following example:

Rat No. 317, having been on TT for 36 days, excreted a urine with a specific gravity of 1·0395 during the first 8 hours of water deprivation. However, the urine collected during the following 16 hours had a specific gravity of 1·0215, a finding which suggested that the urine was not concentrated further because the animal excreted oedema fluid.

B. *Inulin and diodone clearance tests.*

1. *Results obtained on rats fed on carrot diet (CT).*—The results of inulin and diodone clearance tests in a series of 32 rats on CT are shown in Table I in terms of means and their standard deviations. If compared with the results in normal

TABLE I.—*Renal Effects of Protein-deficient Vegetable Diets.*

Type of diet.	Inulin clearance (GFR). M ± S.E. (ml./100 g./min.).	Inulin clearance (GFR). r ± S.E.	Diodone clearance (RPF). M ± S.E. (ml./100 g./min.).	Diodone clearance (RPF). r ± S.E.	Tm_D. M ± S.E. (mg. I/100 g./min.).	Tm_D. r ± S.E.
Standard diet (ST)	0·347 ± 0·0053	− 0·209 ± 0·4082	2·222 ± 0·0581	+ 0·203 ± 0·2001	0·1324 ± 0·00201	− 0·019 ± 0·0110
Protein-deficient "Carrot Diet" (CT)	0·659 ± 0·0602	+ 0·807 ± 0·1851	3·279 ± 0·4510	+ 0·845 ± 0·4231	0·1756 ± 0·03209	+ 0·855 ± 0·2476
Standard diet plus vitamin A	0·748 ± 0·082	+ 0·582 ± 0·2104	4·054 ± 0·3521	+ 0·782 ± 0·3003	0·2885 ± 0·0440	+ 0·808 ± 0·3890
Protein-deficient "Turnip Diet" (TT)	0·640 ± 0·0500	+ 0·703 ± 0·1602	3·384 ± 0·3452	+ 0·803 ± 0·3331	0·0889 ± 0·00898	+ 0·189 ± 0·1938

M ± S.E. = mean and standard error.
r ± S.E. = correlation coefficient between the value indicated and rate of urine flow and standard error of "r."

animals (ST rats) it will be seen that the GFR and RPF of the CT animals was significantly higher (t for GFR = 5·152, $P < 0·001$; t for RPF = 2·330, $P < 0·02$), but that the mean value for Tm_D was much the same (t = 1·550, $P > 0·1$). Calculation of the correlation coefficients between the rate of diuresis and GFR, RPF and Tm_D of the CT animals showed that conclusions drawn from a comparison of means gave an incomplete picture of the significance of the results. These correlation coefficients were all found to be significant (Table I). In other words, it could be shown that, in contrast to normal rats, glomerular filtration rate, effective plasma flow and Tm_D, in the rats on carrot diet, increased with the rate of urine flow.

There was thus no doubt about the difference in renal function between the ST and CT animals, but, in view of the findings of Dicker and Heller (1946) that the kidney function of rats is influenced by an excess of vitamin A in the diet and the fact that the carrot diet contained high amounts of carotene (vitamin A potency = approximately 15,000 I.U./100 g. carrots), it was not clear whether the changes observed in the CT rats could be due to this factor. A comparison (Table I) between the results obtained in rats fed on CT and those in rats fed on standard diet to which an amount of a vitamin A concentrate, approximately equivalent to the vitamin A potency of the carrot diet, had been added, demonstrates the similarity of the mean values of GFR and RPF and the respective correlation coefficients of the two series, but shows a significant difference between the mean values for Tm_D (t = 2·752, $P < 0·01$). This comparison suggested, therefore, that some of the abnormal features of the kidney function of rats fed on CT may have been due to the high vitamin A potency of the diet. However, it indicated also that abnormally low Tm_D values in the CT rats may have been " masked " by the hypervitaminosis.

In order to obtain a clearer picture it was, therefore, decided to perform clearance tests on a further series of rats fed on a diet in which carrots had been replaced by turnips which contain no vitamin A or carotene.

2. *Results obtained on rats fed on turnip diet* (*TT*).—Fig. 2B and Table I show that an increase of GFR correlated with a rise of the rate of urine flow was also encountered in rats whose protein-deficient diet contained no excess of vitamin A potency. The figures for the rate of tubular water reabsorption $(T)_w$ in normal and in hypoproteinaemic rats (Fig. 2) showed further that an increase of urinary water excretion in the normal animals was due to a decrease of tubular water reabsorption, whereas an increase of the rate of urine flow in the TT rats was mainly produced by an increased glomerular filtration rate. Fig. 3 and Table I suggest the possibility that RPF in the TT rats increased with an increase in the rate of urine flow.

So far the findings were similar to those in CT rats. However, the values for Tm_D in the rats on turnip diet showed a very different picture. It will be seen (Fig. 3B) that the majority of the Tm_D values in this series were significantly depressed (t between mean Tm_D of normal rats and mean Tm_D of the animals on TT = 3·929, $P < 0·001$), suggesting that the number of normally secreting tubular units was, in many cases, considerably diminished. Table I shows that Tm_D in this instance was not correlated with the rate of urine flow.

Another change of tubular function noted was a decrease of the rate of tubular chloride reabsorption (T_{Cl}). The mean T_{Cl} in rats on ST was 97·1 $\pm$ 0·25 per cent, that of the rats on TT was 95·5 $\pm$ 0·46 per cent. A statistical comparison

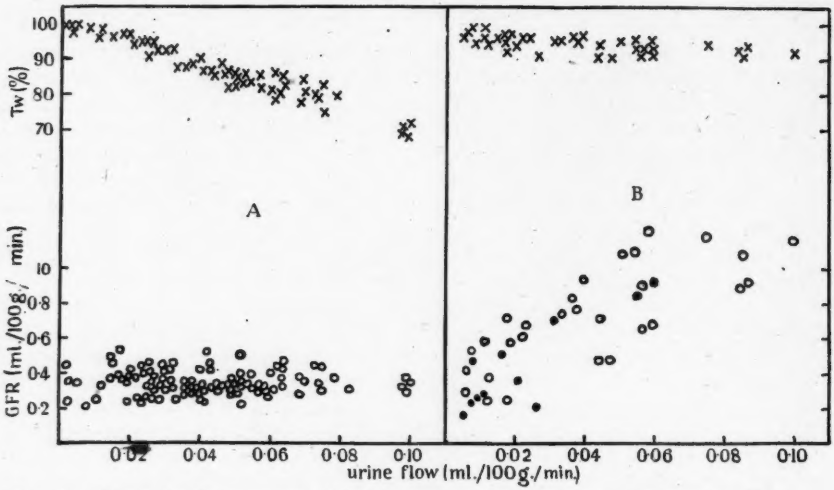

FIG. 2.—Glomerular filtration rate (GFR) and rate of tubular water reabsorption (T_W) in normal rats (A) and in hypoproteinaemic rats on turnip diet (B). ○ = non-oedematous animals. ● = oedematous animals.

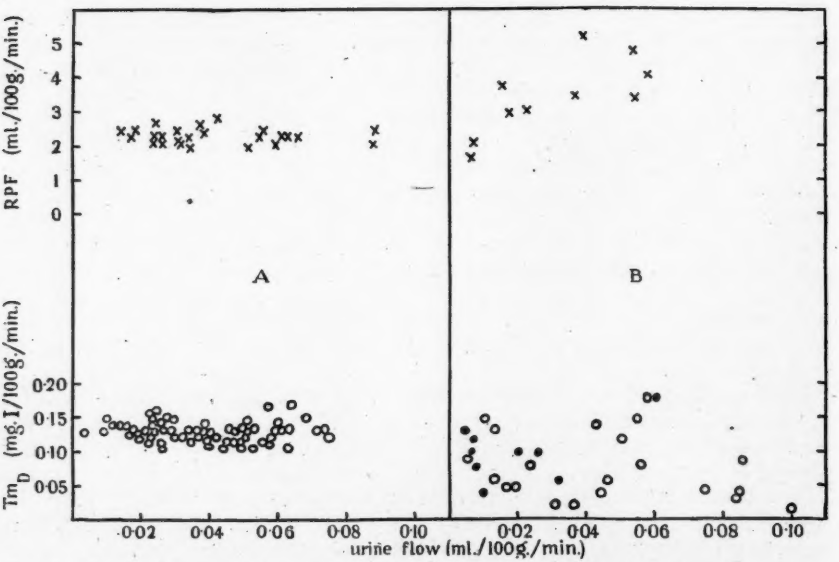

FIG. 3.—Total tubular excretory mass (Tm_D) and diodone clearance at low plasma diodone levels in normal rats (A), and in hypoproteinaemic rats on turnip diet (B). ○ = non-oedematous animals. ● = oedematous animals.

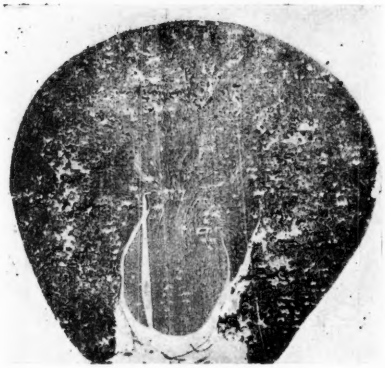

FIG. 4.

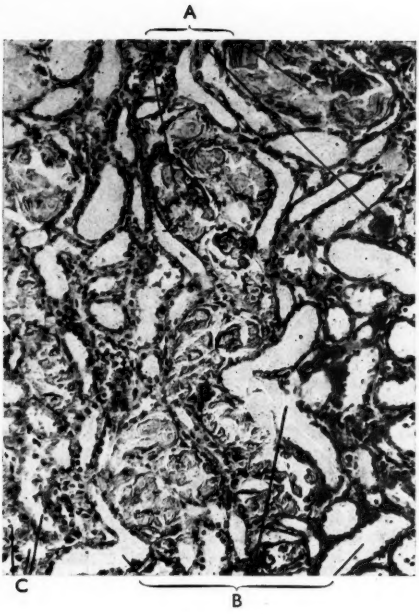

FIG. 5.

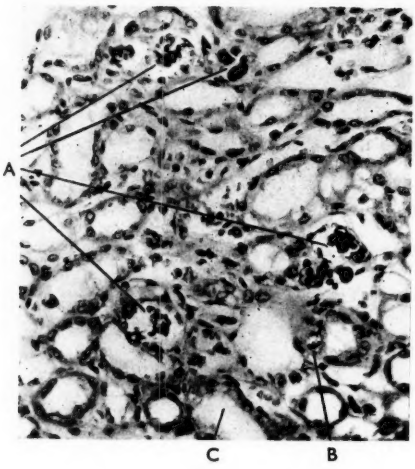

FIG. 6.

FIG. 4.—Transverse hemisection of kidney. The lesions are greatest in the inner cortical zone but extend into both the adjacent zones. The knife marks are due to grains of calcium in the tissue. × 6·7.

FIG. 5.—Transverse hemisection of kidney, showing tubules cut roughly longitudinally. Junction of inner cortical and outer medullary zones. × 143.

A. Calcified debris presumed to be in broad limbs of Henle.
B. Collecting tubules.
C. Straight descending limbs of 1st tubules.

FIG. 6.—Section cut transversely to axis of medullary pyramid at junction of inner cortical and outer medullary zones. × 284.

A. Necrotic and calcifying broad limbs of Henle.
B. Early calcification of necrotic cell in wall of a broad limb of Henle.
C. Descending straight limb of 1st tubule.

Dicker, Heller and Hewer.

showed that the difference was significant ($t = 2{\cdot}992$, $P < 0{\cdot}01$). It should be added that the urine of the protein-deficient rats contained neither sugar nor protein.

Gross Post-mortem Findings.

The only external sign of oedema was the occasional occurrence of oedema of the penis. Ascites or oedema of the abdominal wall tissue was found in 40 out of 68 rats on the carrot diet, but in only 13 out of 46 on the turnip diet. It was noted that of the remainder, without evident oedema, some had moist tissues while others seemed abnormally pale and dry.

Ascitic fluid found in rats on the carrot diet had a milky appearance which may have been due to badly soluble calcium salts which were abundantly present. Ascitic fluid from rats on turnip diet showed less turbidity. The mean protein content of nine samples of ascitic fluid of rats on turnip diet was $1{\cdot}01 \pm 0{\cdot}062$ (S.E.) g./100 ml.

None of the thoracic or abdominal organs showed any gross changes. In particular the liver appeared entirely normal.

The kidneys also showed no recognizable change in the gross. The kidneys, liver and spleen were weighed accurately and assessed as percentages of the body weight. The mean kidney weight and S.E. of rats on TT was $1{\cdot}06 \pm 0{\cdot}030$ per cent (54); that of controls on ST was $1{\cdot}05 \pm 0{\cdot}045$ per cent (46); $t = 0{\cdot}185$, $P > 0{\cdot}9$.

The mean liver weight of TT rats was $4{\cdot}70 \pm 0{\cdot}108$ per cent (46); that of the controls was $4{\cdot}40 \pm 0{\cdot}074$ per cent (37). The difference is just significant. ($t = 2{\cdot}290$, $P < 0{\cdot}05$.)

The mean spleen weight of TT rats was $0{\cdot}55 \pm 0{\cdot}034$ per cent (46); that of the controls was $0{\cdot}50 \pm 0{\cdot}024$ per cent (37). $t = 1{\cdot}201$ $P > 0{\cdot}2$.

Histological Findings.

Liver.—In no case were any histological lesions found in the liver.

Kidney.—In all kidneys where lesions were found they were of the same character, differing only in degree from case to case.

In no instance was any change detected in the glomeruli, but the tubules had suffered very extensive necrosis and calcification. In the more severe examples the calcification was so great that good sections could not be cut without decalcification of the block (Fig. 4); in such cases it was often impossible to identify with certainty the anatomical parts of the tubules that were affected. As an aid to identification we employed the method advocated by McFarlane (1941), dividing the kidney into four zones: outer and inner cortex and outer and inner medulla. The predominant feature in the outer cortex is the first convoluted tubule; in the inner cortex the descending straight limb of the first tubule; in the outer medulla the broad limb of Henle; and in the inner medulla the collecting tubules.

Using transverse hemisections of the kidney with tubules cut longitudinally together with sections cut transversely to the axis of the medullary pyramids, and comparing them with normal controls, we were able to locate the early lesions accurately.

The first convoluted tubules, their descending straight limbs, and the narrow limbs of Henle appeared intact, but the broad limbs of Henle had become necrotic and calcified (Fig. 5 and 6). Some of the calcified debris seemed to have passed

on into the lumen of the second convoluted tubules, but the latter were not themselves involved in the necrotizing process. Apart from an inconstant degree of dilatation the collecting tubules were intact. There were no haemorrhages and no vascular changes. No interstitial inflammatory reaction was seen. The pelves were uniformly clean. Traces of fat were seen in the necrotic material but not elsewhere.

Incidence of the Kidney Lesions.

Of the 33 rats on TT diet whose kidneys were examined, severe lesions were found in 12, moderate in 9, and none in the remaining 12.

Of the 51 rats on CT diet 33 had severe renal lesions, 13 moderate and the remaining 5 showed no lesions.

No relationship was found between the incidence or degree of histological lesions and the impairment of Tm_D. This lack of correlation of the results agrees with the fact that it is as yet impossible to localize the various renal functions in the tubules with certainty.

DISCUSSION.

Renal Function Tests.

1. A general increase of the rate of glomerular filtration might have been expected from the decrease of the plasma colloid osmotic pressure, but no clear correlation between the level of the plasma proteins and the glomerular filtration rate was established. This finding agrees with the results of Eggleton, Pappenheimer and Winton (1940), who showed that, in the chloralosed dog, diuresis due to dilution of the plasma proteins was not accompanied by a significant rise of the creatinine clearance (= GFR). However, while no correlation between the plasma protein level and the GFR of the hypoproteinaemic rats could be demonstrated, it could be shown that the GFR was correlated with the rate of urine flow. Such a correlation is absent in normal rats (Dicker and Heller, 1945).

When the rate of tubular water reabsorption (T_w) in the rats on the protein-deficient "turnip diet" was calculated from the values for GFR, it became obvious that T_w remained practically constant throughout the wide range of urinary excretion rates encountered in the experiments. It seems, therefore, that the protein-deficient rats responded to an extra water load, not by a decrease in the rate of tubular water reabsorption as do normal animals, but by a rise in the rate of glomerular filtration. The observation that protein-deficient rats on turnip diet seemed unable to dilute the urine to the same degree as normal animals suggests the possibility that this "glomerular regulation" of water diuresis may have been due to an impairment of tubular function. The occurrence of histologically demonstrable tubular lesions in these protein-deficient animals renders this hypothesis more likely.

By what mechanism was the "glomerular diuresis" achieved? A definite answer cannot be given, but it should be noted that determinations of the diodone clearance at low plasma diodone levels yielded values which, if interpreted as a measure of the effective renal plasma flow (RPF), suggest that a rise of GFR was correlated with a rise of RPF. Such an increase of RPF would account for the parallel rise of GFR.

2. Determinations of the "total tubular excretory mass" (Tm_D) established

the fact that, in the majority of the protein-deficient animals on turnip diet, at least one tubular mechanism, viz. that concerned with the transfer of diodone, was seriously deficient. This deficiency was demonstrated by the significant depression of the Tm_D values. Similarly low Tm_D values, interpreted as indications of a reduction of functional renal parenchyma, have been observed in cases of human chronic glomerulonephritis (Earle, Taggart and Shannon, 1944), and more recently (Mollison, 1946) in a case of oedema with hypoproteinaemia (serum proteins = 4·0 g./100 ml.) but without albuminuria.

It will be noticed that Tm_D, in the protein-deficient animals, was usually calculated as per 100 g. rat. The objection could be made that the severe loss of body weight of such animals renders this method of calculation inaccurate if the normal relation of body weight to kidney weight is disturbed. However, the mean kidney weight of the TT rats was not significantly increased (see p. 165). There seemed, therefore, to be no need to modify the mode of calculation of Tm_D.

Two other factors which modified the diodone clearance of hypoproteinaemic animals will have to be considered: the increased water content of the plasma, and the decrease of "bound" diodone resulting from the diminished plasma protein concentration. Both factors increase the proportion of diodone filterable by the glomeruli, but the expressions for both of them (W and F) appear in the formula for the calculation of Tm_D. The validity of the Tm_D values obtained is, therefore, not impaired. However, these factors are likely to have had some influence on the values for RPF which are directly based on diodone clearance, though this influence should be negligible compared with the depression of the clearance due to an impairment of the diodone transfer mechanism. These considerations suggest that the figures given for RPF represent only relative values, but they still leave the finding that the diodone clearance at low plasma diodone levels was correlated with the rate of urine flow.

3. A result which has so far not been discussed is the abnormal response of rats on the protein-deficient vegetable diets to a standard amount of water given by stomach tube. There would seem to be at least two likely explanations for the decreased diuretic response observed in these animals: (*a*) the apparent failure of the renal tubules to reduce the rate of water reabsorption as a response to the increased plasma water load, a failure only partly compensated by the increased glomerular filtration rate; (*b*) an increased "preparedness" of extrarenal tissues to retain water (Volhard's Oedembereitschaft). If the first possibility obtained, a dilution test would, as usually assumed by clinical investigators, give a true indication of the functional capacity of the renal tubules; however, an increased tendency of the tissues to retain water would withhold a fraction of the administered fluid from the kidneys, and lead to the production of a less diluted urine owing to a decrease of the plasma water load. It is clear that if either or both possibilities apply, the overall result would be a diminished water diuresis and the retention of water in the body.

Cases of oedema, as manifested by ascites or by accumulation of fluid in the extraperitoneal tissue, were not infrequently encountered in protein-deficient rats which had received water by stomach tube. Ascites was also found in animals without an extra water load. The presence of oedema seemed to have no bearing on the outcome of the renal clearance tests. Results obtained in these animals were not significantly different from those in rats on protein-deficient vegetable diets without visible oedema.

Histological Findings.

Dunn and Polson (1926) found very similar lesions restricted to the broad limb of Henle in experimental uric acid nephritis, and McFarlane (1941) showed that the damage in phosphate nephritis has the same distribution. These are the only experimental nephropathies hitherto described in which the lesions are confined to the broad limb of Henle.

Since this work was completed Morehead, Fishman and Artom (1945) have reported the experimental production of tubular necrosis and calcification in rats maintained on a stock diet supplemented with serine by stomach tube. This necrosis was intensified if the rats were on a diet deficient in protein and the B group of vitamins, and some necrosis was observed in rats on the deficient diet without the addition of serine ; but the low protein diet in these experiments was deficient in choline as well, and the lesions seem to have been more diffuse than they were in our series of rats.

Although calcification was the most immediately striking feature of the kidney lesions in our protein-deficient rats, there is little doubt that the primary change was necrosis of the tubular epithelium. There is a great tendency for necrotic renal epithelium to become calcified in experimental animals, and Hepler and Simonds (1945) investigated the possible role of phosphatase in this process. In their studies on dogs with chemically-induced renal necrosis they found that the degree of calcification was related to the nature of the chemical poison employed and not to the phosphatase content.

The absence of liver lesions is not surprising, since Himsworth and Glynn (1944) found that there was no necrosis when the diet contained less than 4 per cent casein.

General.

The diets which have been shown to produce lesions of the renal tubules and renal functional changes differed in several respects from the experimental protein-deficient diets in more common use : the casein and yeast proteins of the control diet were completely replaced by carrots or turnips which contain less than 1 per cent protein, but sufficient choline to eliminate a deficiency of that substance (Engel, 1943).

It would seem from a report of Mollison (1946), published after completion of our experiments, that impairment of kidney function similar to that described in the present paper may occur in human beings on a similar diet resulting in a similar degree of hypoproteinaemia.

SUMMARY.

1. The kidney function of adult rats fed on protein-deficient vegetable diets was investigated. Kidney function tests were performed when the rats had been on protein-deficient vegetable diets for about 36 days, and when their plasma protein concentration had decreased from 7.58 ± 0.682 to 4.26 ± 0.097 g./100 ml.

2. The results of inulin clearance determinations showed that, in contrast to normal rats, the glomerular filtration rate of the protein-deficient rats was significantly correlated with the rate of urine flow.

3. Estimations of the total tubular excretory mass (Tm_D) of rats on a protein-deficient " turnip diet " gave figures which were significantly lower than those in normal rats, showing an impairment of the tubular diodone transfer mechanism.

4. Urinary concentration and dilution tests gave the following results. The urine of rats on standard diet reached a mean specific gravity of 1·070 ± 0·0037 after 24 hours of water deprivation. The same series of animals at the end of the period of protein-deficient feeding excreted a urine of the mean specific gravity of 1·041 ± 0·0044. The mean maximum dilution of the urine (in terms of specific gravity) of normal rats which had received 5 per cent of their body weight of water was 1·005 ± 0·0005, that of the same animals when protein-deficient was 1·010 ± 0·0016.

5. The results of the concentration and dilution tests are discussed with reference to the occurrence of visible oedema in the animals on protein-deficient vegetable diets and to the decreased water diuresis usually observed in hypoproteinaemic rats.

6. No evidence was obtained for a relation between the incidence of visible oedema and the results of the clearance estimations.

7. Renal lesions were found in many of the rats on protein-deficient vegetable diets. They consisted of necrosis and calcification of the broad limbs of Henle, the glomeruli and other parts of the nephron showing no apparent change.

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OBSERVATIONS ON THE ACIDOPHIL CELL CHANGES IN THE PITUITARY IN THYROXINE DEFICIENCY STATES. I. ACIDOPHIL DEGRANULATION IN RELATION TO GOITROGENIC AGENTS AND EXTRATHYROIDAL THYROXINE SYNTHESIS.

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THYROID ablation in rats induces changes in the cytology of the anterior pituitary. These changes were described briefly by Severinghaus, Smelser and Clark (1934), and in more detail by Zeckwer, Davison, Keller and Livingood (1935). The changes affect both types of chromaffin cells. There is a marked increase in the numbers and size of the basophil cells (" thyroidectomy cells ") which makes these cells the dominant feature of the thyroidectomy pituitary. The acidophil cells on the other hand lose their acidophil granules, and become almost indistinguishable in histological preparations from chromophobes. The significance of these cell changes has not yet been correctly explained, and the fact that two cell changes are obtained after the elimination of the thyroid hormone complicated the earlier attempts to locate the site of thyrotropin production in the pituitary (Zeckwer, Davison, Keller and Livingood, 1935 ; Marine, Rosen and Spark, 1935 ; Griesbach and Purves, 1943). Subsequently Griesbach and Purves (1945) have presented evidence showing that the basophil cells are the producers of the thyrotropic hormone, and that their prompt increase in numbers under conditions of the slightest thyroxine deficiency is due to a highly sensitive homeostatic mechanism affecting the pituitary thyroid relations. At the same time it was noted that degranulation of acidophil cells coincided with a state of extreme thyroxine deficiency.

The present paper deals with investigations into the relationship between extreme thyroxine deficiency and degranulation of the acidophil cells of the pituitary. In these experiments the thyroxine formation in the animal has been controlled by surgical ablation of the thyroid alone, and in combination with administration of thiourea which inhibits thyroxine synthesis. The effect of iodide in doses much above the physiological level has also been investigated.

METHODS.

The rats used were from a Wistar strain which had not been maintained by inbreeding. Rats of both sexes aged 8–10 weeks at the beginning of experiments were used. Thyroidectomy was performed as previously described (Griesbach and Purves, 1945), and 0·25 per cent. of calcium chloride added to the drinking

water after operation. The supply of calcium reduced the number of post-operative deaths from tetany. Thiourea was given as a 0·25 per cent. solution in the drinking water. When potassium iodide was injected, 0·1 ml. of 1·3 per cent. solution in normal saline was given daily. Since the rats were about 100 g. in weight, these injections supplied approximately 1·0 mg. of iodine per 100 g. body weight per day. To prevent the formation of free iodine in these solutions sodium bicarbonate (0·05 per cent.) was added to render the solution alkaline.

The histological examination of the pituitaries and the enumeration of the three categories of cells in the pituitaries were carried out as previously described (Griesbach, 1941 ; Griesbach and Purves, 1943).

A stock solution of thyroxine containing 10 microgrammes of *dl*-thyroxine per ml. was prepared. Its composition was :

dl-thyroxine . .	0·001 g.
Sodium carbonate .	0·020 g.
Sodium chloride . .	0·85 g.
Water to . . .	100 ml.

The solid constituents were dissolved by boiling with part of the water to form a clear solution. After cooling the solution was made up to volume with freshly boiled distilled water. This solution was kept in the refrigerator and diluted with saline as required to make a solution containing 1·0 microgramme per ml. The rats were weighed three times weekly, and the dosage of thyroxine adjusted to maintain constant dosage per 100 g. body weight. Subsequent references in this paper to thyroxine dosage refer to the dosage per 100 g. body weight.

The diet used was that described as S3 in a previous paper (Griesbach, Kennedy and Purves, 1945). It consisted of bran 30 per cent., pollard 25 per cent., bone meal 15 per cent., pea meal 15 per cent., and maize meal 15 per cent.

RESULTS.

I. Total Thyroidectomy.

Ia. Rats that were sacrified four weeks or more after total thyroidectomy had pituitaries in which the acidophil cells had undergone complete degranulation. The acidophil cells do not disappear, but can be recognized in many places in their degranulated condition by their appearance and characteristic distribution along capillaries. The lack of the acidophil granules, which are highly refractile in the fresh material, results in a change in the naked-eye appearance of the pituitaries in the unfixed state. Such degranulated pituitaries are more transparent than the normal pituitary, which has a more porcellaneous appearance. The absence of red staining material, except for red blood corpuscles, in the Mallory stained section results in a blue coloured section, instantly distinguishable from sections of normal pituitaries stained in the same way. When the progress of the degranulation process was studied by sacrificing animals at intervals after operation, it was found that a reduction in the acidophil substance of the individual cells was evident after 7 days. After 14 days there was a law percentage count of the acidophil cells because a substantial proportion of these cells had been completely degranulated. Those which still contained acidophil granules had

their content of acidophil granules much reduced, the remaining granules forming scanty rings or crescents round the nucleus. After 21 days degranulation of acidophil cells was complete in all animals.

Ib. Total thyroidectomy plus thyroxine injections. Prevention of degranulation.

When thyroxine injections were administered from the date of thyroidectomy degranulation of acidophil cells was prevented. The amounts of acidophil substance retained varies with the dosage of thyroxine. When 0·1 microgram of *dl*-thyroxine per 100 g. body weight was administered daily for four weeks after thyroidectomy a small but definite protective action on the acidophil granules was observed. When 0·25 microgram of *dl*-thyroxine per 100 g. was administered daily a substantial proportion of the acidophil granules was retained, and the counts of granulated acidophil cells were about one-half of the normal values. The daily administration of 0·5 microgram of *dl*-thyroxine per 100 g. sufficed to retain the acidophil cells in approximately normal condition, both in the proportionate numbers of granulated cells and in the content of acidophil substance in the individual cells.

Ic. Regranulation.

Thyroxine injections begun four weeks after thyroidectomy, i.e. when degranulation was complete, resulted in the reappearance of acidophil granules. When thyroxine injections were begun four weeks after total thyroidectomy and continued for three weeks the results were as follows: 0·1 microgram *dl*-thyroxine per day resulted in a scanty, almost insignificant regranulation; 0·25 microgram per day resulted in the regranulation of about one-quarter of the acidophil cells; while 0·5 microgram per day restored the granules to somewhat more than one-half of the acidophil cells. Thus after degranulation had taken place a given dose of thyroxine was less effective in restoring the granulation than the action of the same dose administered from the date of operation in retaining the acidophil granules.

II. Total Thyroidectomy plus Iodide.

IIa. Prevention of Degranulation.

When iodide injections were begun before thyroidectomy and continued after the operation, or were begun from the date of operation, there was a protective

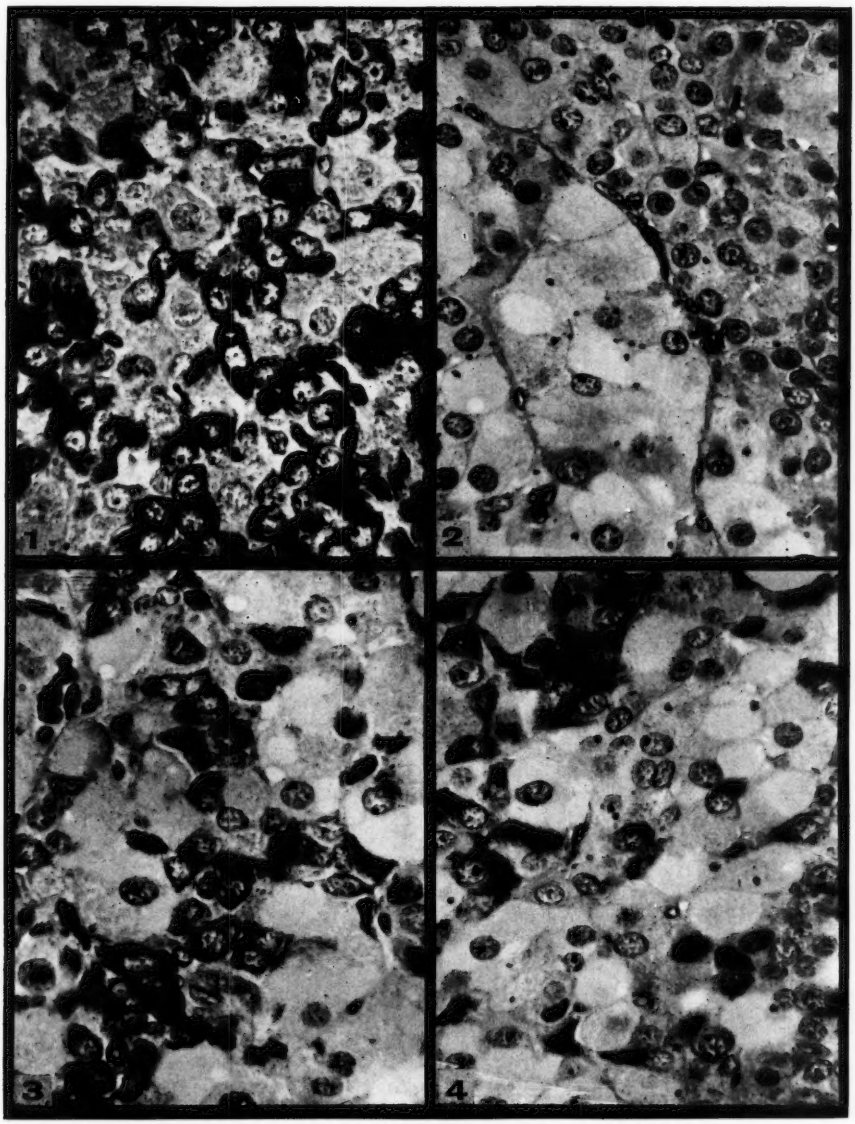
DESCRIPTION OF PLATE.

FIG. 1.—Pituitary of normal rat. The acidophil cells, stained red in the section, are reproduced here with dark or even black cytoplasm. The large grey granular cells are normal basophils. Chromophobe cells are represented only as faintly stained nuclei. Haematoxylin and Mallory × 650.

FIG. 2.—Pituitary of rat four weeks after thyroidectomy. Lower left a collection of "thyroidectomy" cells showing accumulations of clear hyaline substance. Upper right, chromophobes and degranulated acidophils. Having lost their acidophil granules the latter do not appear dark in the photo, and are only distinguished from chromophobes by their more abundant cytoplasm. Haematoxylin and Mallory × 650.

FIG. 3.—Pituitary of rat four weeks after thyroidectomy. 0·5 micrograms of *dl*-thyroxine was injected daily from the date of operation. "Thyroidectomy" cells as in fig. 2. Many acidophils retaining their acidophil granules (dark in the photo). Haematoxylin and Mallory × 650.

FIG. 4.—Pituitary of rat four weeks after thyroidectomy. 1·3 mg. of potassium iodide were injected daily from date of operation. "Thyroidectomy" cells as in figs. 2 and 3. Many acidophil cells have retained their granules as in fig. 3. Haematoxylin and Mallory × 650.



Purves and Griesbach.

action on the acidophil granules similar to that exerted by small doses of thyroxine. The daily injection of 1 mg. of iodine as iodide resulted in the retention of acidophil granules in approximately one-half of the acidophil cells. The effect therefore of this dosage was similar to that produced by 0·25 micrograms of *dl*-thyroxine per day.

IIb. Regranulation.

Rats were thyroidectomized and kept for 21 days. Sacrifice of a proportion of the animals then confirmed that acidophil degranulation was complete. At this time iodide injections (1·0 mg. of iodine daily as potassium iodide) were begun and continued for 28 days. Examination of the pituitaries showed that regranulation of the acidophil cells had taken place, the cell counts showing granulated acidophil cells in about one-third of the normal numbers. Iodide injections therefore had a restorative action on the acidophil granules similar to the action of small injections of thyroxine. As in the case of the thyroxine injections, the amount of granulation produced by the restorative action of iodide injections was less than that found in animals in which the granules had been preserved by injections begun before thyroidectomy.

III. Thiourea Alone.

As we have previously described, the enlargement and the conspicuous appearance of the basophil cells of the pituitary makes the sections of the pituitary of the rat receiving thiourea appear deficient in acidophil cells. Cell counts, however, show that acidophils are present in normal or nearly normal numbers. We have not observed extensive degranulation of pituitary acidophil cells in any rats treated with thiourea alone, even after many months' treatment.

Thyroxine in amounts equal to 2·25 micrograms of *dl*-thyroxine per 100 g. body weight per day prevented the basophil changes as previously reported (Griesbach and Purves, 1945), and thus prevented the appearance of any thyroxine deficiency changes. Smaller doses of thyroxine did not affect the pituitary appearance, since the acidophil cells were well granulated withoutt hyroxine injections.

IV. Thiourea plus Iodide.

IVa. Daily iodide injections (1·0 mg. of iodine as iodide) were given simultaneously with the administration of thiourea in the drinking water. After four weeks complete degranulation of acidophil cells was found in all animals. The appearance was identical with that produced by total thyroidectomy, i.e. total thyroxine deficiency.

IVb. The effects of thyroxine injections in these animals were identical with those obtained after total thyroidectomy. Injections of 0·25 micrograms of *dl*-thyroxine per day retained the granulation in about one-half of the acidophil cells, while 0·5 micrograms sufficed to maintain the acidophil cells in normal numbers and appearance.

IVc. The restorative action of thyroxine injections on the acidophil granules after degranulation had taken place was decidedly less than the protective action

of the same doses given from the institution of the thiourea-iodide treatment. Thus, in one experiment 0·25 micrograms of thyroxine per day was practically without effect on the degranulated acidophil cells in six out of seven animals. In the seventh animal there was regranulation to about one half of the normal. Injections of 0·5 micrograms of *dl*-thyroxine restored granules to only about two-thirds of the acidophil cells. This relative ineffectiveness of the restorative action of thyroxine injections was similar to that found after thyroidectomy.

V. Total Thyroidectomy plus Thiourea.

The appearance of basophil activation and acidophil degranulation observed after thyroidectomy alone was not altered in any respect by the administration of thiourea to thyroidectomized animals. The time course of degranulation was the same as in thyroidectomized animals not receiving thiourea. Thyroxine injections protected against degranulation in the same dosage as after thyroidectomy alone and the restorative action of thyroxine injections on degranulated acidophil cells was also quite unaffected by the addition of thiourea.

VI. Total Thyroidectomy plus Iodide plus Thiourea.

VIa. Complete degranulation of acidophil cells ensued in three weeks in animals that were thyroidectomized and given thiourea in the drinking water and iodide by injection (1·0 mg. of iodide as potassium iodide per day). The thiourea administration therefore abolished the protective action of iodide which was observed on totally thyroidectomized animals not receiving thiourea.

VIb. The effect of thyroxine injections on totally thyroidectomized animals receiving iodide plus thiourea was the same as in thyroidectomized animals not receiving iodide and thiourea. Injections of 0·25 micrograms of *dl*-thyroxine daily maintained about one half of the acidophil cells in the granulated condition, while 0·5 micrograms sufficed to maintain the acidophil cells in normal condition.

VIc. Regranulation of the acidophil cells in which degranulation had taken place under the above regime required larger doses of thyroxine than those given above to produce the same amount of granulation. Thus 0·25 micrograms of *dl*-thyroxine daily for three weeks restored granules to about one-quarter of the acidophil cells, while 0·5 micrograms restored granules to about two-thirds of the cells. The regranulation effect of thyroxine injections was therefore unaffected by administration of iodide plus thiourea, since the regranulation effect was comparable to that observed in animals in which degranulation had been produced by thyroidectomy alone.

In all of these observations the reactions of male and female animals were studied separately and the responses of the two sexes were found to be substantially identical. Moreover, most of the observations were repeated many times and the average responses of the groups were quite consistent. However, although the average group responses were regular, there was some individual variation in the amount of acidophil granulation in the animals of those groups in which partial degranulation was present. This was particularly noticeable in those experiments in which regranulation of degranulated acidophil cells was produced, as in these groups occasional animals showed a relative failure to regranulate under the influence of thyroxine or iodide injections.

TABLE I.—*Pituitary Acidophil Cell Granulation in Thyroxine Deficiency States.*

Summary of Results.

Group number and experimental treatment.	A. Without thyroxine.	B. Thyroxine injections for granule preservation.		C. Thyroxine injections for regranulation.	
		0·25μg.	0·5 μg.	0·25 μg.	0·5 μg.
I. Thyroidectomy	0	++	+++	+	++
II*a*. Thyroidectomy + KI 1·3 mg./day	++	..	..	..	..
II*b*. Thyroidectomy 4 wks., then KI 1·3 mg./day	+	..	..	..	..
III. Thiourea	+++	..	..	..	..
IV. Thiourea + KI 1·3 mg./day	0	++	+++	+	++
V. Thyroidectomy + thiourea	0	++	+++	+	++
VI. Thyroidectomy + thiourea + KI 1·3 mg./day	0	+++	+++	+	++(+)

Column B gives results obtained by thyroxine injections from commencement of experiment.

Column C gives results obtained by injecting thyroxine for 3 weeks, beginning 4 weeks after commencement of experiment.

The amount of acidophil cell granulation is represented by plus signs, which may be related to the percentage cell counts of the pituitaries as under :

+++ indicates approximately 36 per cent or more granulated acidophil cells.
++ „ „ 24 per cent granulated acidophil cells.
\+ „ „ 12 per cent „ „
0 „ „ No „ „

Basophil cell changes were present in all pituitaries.

DISCUSSION.

Acidophil Cell Response to Thyroxine Deficiency.

The fact that thyroidectomy changes in the rat's pituitary can be prevented by administration of synthetic thyroxine (Hohlweg and Junkmann, 1935) makes it certain that both the cytological changes appearing in the rat's pituitary after thyroidectomy are due to thyroxine deficiency. In a previous paper on the significance of the basophil cell changes in thyroxine deficiency states (Greisbach and Purves, 1945), we showed that basophil changes in the pituitary were produced by slight degrees of thyroxine deficiency which did not affect the numbers, appearance, or extent of granulation of the acidophil cells. The correspondence between the increase in numbers and size of the basophil cells and the histological activation of the animal's own thyroid tissue led to the deduction that these conspicuous basophil cells (the so-called "thyroidectomy" or "goitre" cells) are the site of thyrotropin formation, and their prompt response to falling thyroxine levels constitutes a homeostatic mechanism which, in the presence of functioning thyroid tissue, acts to maintain the blood thyroxine concentration at a constant level.

The results obtained in the present investigation show that degranulation of pituitary acidophil cells in thyroxine deficiency occurs only as the result of complete thyroxine deprivation. Acidophil degranulation occurred regularly

after thyroidectomy (Result I*a*), provided that the removal of the thyroid was complete. In those animals in which a portion of thyroid tissue had been inadvertently or purposely left at operation no acidophil degranulation occurred. The results of thyroxine injections (Result I*b*) show that 0·5 microgram of *dl*-thyroxine per day is necessary to maintain the acidophil cells in a normal state. Since the optically active *l*-thyroxine which is the product of natural thyroxine synthesis is probably twice as active as the racemized *dl*-thyroxine used for injections (Reineke and Turner, 1945), a fragment of thyroid synthesizing 0·25 micrograms of *l*-thyroxine per day would suffice to maintain the acidophils in normal condition.

The retention of acidophil granules after thyroidectomy is a sensitive test for thyroxine, since the effect of as little as 0·1 micrograms of *dl*-thyroxine per day is discernible. Injections of 0·25 micrograms of thyroxine per day suffice to retain granules in about one-half of the acidophil cells, so that for amounts of thyroxine of this order the response of the acidophil cells would provide a means of assay of thyroxine-containing substances provided the specificity of the reaction could be established.

After degranulation had taken place, approximately twice as much thyroxine was needed for the restoration of the granules as was required to retain them (*cf.* Result I*b* with I*c*), so that in these experiments in which four weeks elapsed between thyroidectomy and the beginning of thyroxine injections, and in which degranulation was complete for only about one week the action was not strictly reversible. This suggests that thyroxine deprivation of the degree associated with acidophil degranulation results in damage to the acidophil cells.

Severinghaus (1942) has shown that in rats thyroidectomized at birth irreversible degenerative changes take place in the pituitary cells which cannot be repaired after the lapse of six weeks by thyroxine administration. Such pituitaries show an absence of acidophil and of basophil cells. Severinghaus concludes that the pituitary cells need thyroxine to enable them to carry out their normal functions, and that a prolonged period of thyroxine deprivation results in irreversible damage to the cells, which never regain their function later although thyroxine be subsequently supplied. The relative difficulty of regranulation in our rats is consistent with Severinghaus' explanation of the thyroxine deficiency effects on the pituitary.

Extrathyroidal Thyroxine Synthesis.

The action of iodide injections in preventing degranulation of acidophils in the thyroidectomized rat is similar to that of small injections of thyroxine (Result III*a*). This result is considered to be due to the synthesis of substances with thyroidal activity in tissues other than the thyroid. It is probable that it is thyroxine itself that is thus formed, since the studies of Morton, Chaikoff, Reinhardt and Anderson (1943) carried out with radio-active iodine have shown the formation of minute amounts of thyroxine from injected radio-active iodine in thyroidectomized rats. Our observations have indicated that daily injections of one milligram of iodine as iodide have approximately the same effect on the pituitary histology as have daily injections of 0·25 micrograms of *dl*-thyroxine. Assuming that optically active *l*-thyroxine is twice as active physiologically as racemic thyroxine, this would indicate the formation of 0·125 micrograms

of *l*-thyroxine per day, which would contain 0·083 micrograms of *l*-thyroxine iodine. Thus our observations indicate that 0·0083 per cent of the milligram doses of iodine which we injected daily were converted to *l*-thyroxine. The indicated conversion is of the same order as that calculated from the data given by Morton, Chaikoff, Reinhardt and Anderson (1943) in their Table III, which presents the results obtained with tracer doses of radio-active iodine in rats in which the completeness of thyroid removal was exhaustively tested. On the assumption that extrathyroidal thyroxine synthesis proceeds at a rate approximately proportional to iodide concentration, the amount of thyroxine formation expected in our thyroidectomized rats, which received from dietary sources about 4 micrograms of iodine per day, would be about 0·0005 micrograms—an amount which is insignificant.

The formation of thyroxine from the injected iodide solutions cannot be due to iodination of tissue proteins by free iodine in the solutions used, because chemical tests showed the absence of free iodine in these solutions. Moreover, the addition of small amounts of reducing agents such as thiosulphate or sulphite to the solution was without effect on the results obtained, so that the thyroxine formed must have been derived from iodine supplied in the form of iodide.

The remarkable consistency with which degranulation takes place after thyroidectomy shows that if ectopic thyroid tissue does occur in our strain of rats it must be in amounts too small to secrete physiologically significant amounts of thyroxine. With regard to the increased extrathyroidal synthesis of thyroxine after iodide injections in thyroidectomized animals, we have further evidence which will be produced in a subsequent paper that this cannot be due to the presence of ectopic thyroid tissue. At the present juncture we would merely draw attention to the regularity with which the iodide injections preserve the acidophil granules in thyroidectomized animals' pituitaries, which indicates approximately the same amount of thyroxine synthesis in each animal, a result inconsistent with the hypothesis that this synthesis is due to ectopic thyroid tissue, the occurrence of which would be expected to be erratic.

Inhibition of Extrathyroidal Thyroxine Synthesis by Thiourea.

The administration of thiourea to totally thyroidectomized rats (Result V) did not modify the behaviour of the pituitary cells. Thyroxine injections had the same protective and restorative action on the acidophil granules as in animals not receiving thiourea. When therefore the thyroidectomized animals receiving iodide plus thiourea show complete degranulation (Result VI*a*), we conclude that the thiourea has inhibited the extrathyroidal synthesis of thyroxine from injected iodide which would have appeared in thyroidectomized rats receiving iodide alone (Result II*a*).

Synergism of Iodide and Thiourea.

The administration of thiourea to the intact animal does not in our experience cause extensive degranulation of acidophil cells. This indicates that thyroxine secretion from the thyroid still continues at a sufficient level to maintain the acidophils in a normal appearance. This corresponds to the findings of Leblond and Hoff (1944), who found that both thiourea and thiouracil produced in the pituitary the appearance observed in cases of partial thyroidectomy. The effect of simultaneous administration of iodide and thiourea (Result IV*a*) is therefore of great

interest because the degranulating effect obtained here cannot be obtained by the administration of either of these substances alone. The administration of thyroxine in such animals has exactly the same granulation protecting and granulation restoring action as in thyroidectomized animals (Result IV*a* and *c*), so that it seems clear that the response of the acidophil cells is not modified by the thiourea and iodide treatment, and their behaviour can be used as a valid indication of the thyroxine level. A comparison of Results IV*a*, *b* and *c* with Results VI*a*, *b* and *c* shows that when iodide and thiourea are given, the presence or absence of the thyroid is quite without effect on the appearance of the acidophil cells, which are, however, clearly responsive to thyroxine administered by injection. This makes it certain that thyroxine secretion from the thyroid gland under the combined influences of iodide and thiourea is reduced to completely insignificant levels. There is therefore a remarkable synergism between thiourea and high iodide concentration in inhibiting thyroxine synthesis in the thyroid. This is probably of great significance in relation to the problem of the site and mode of action of the thiourea on the thyroid, although its meaning is at present obscure.

SUMMARY AND CONCLUSIONS.

(1) The basophilic cell increase, and the acidophil cell degranulation which constitute the complete reaction of the mammalian pituitary to thyroidectomy can be dissociated under conditions of controlled thyroxine deficiency. Thus while the basophil cell changes appear in response to slight degrees of thyroxine deficiency, the degranulation of the acidophil cells appears only in response to complete thyroid deficiency.

(2) After interruption of the thyroxine supply of the immature rat by thyroidectomy, degranulation of the acidophil cells proceeds at variable rates in individual animals, but is virtually complete in all animals after three weeks.

(3) After thyroidectomy, injections of thyroxine prevent the degranulation reaction of the pituitary acidophil cells. The amount of thyroxine required to retain the acidophils in normal condition is about 0·5 micrograms of *dl*-thyroxine per 100 grammes per day, which is about one-fifth of the normal thyroxine requirement of our rats. Smaller doses of thyroxine result in partial retention of acidophil granulation.

(4) Thyroxine injections begun shortly after acidophil degranulation is complete will restore the granules to the acidophil cells. However, some degree of impaired function seems to be produced by conditions of severe thyroxine deficiency, since larger doses of thyroxine are required to restore the granules than would be required to retain them if given from the date of thyroidectomy.

(5) Injections of large amounts of iodide in thyroidectomized rats have a protective action on the pituitary acidophil granules similar to that of small amounts of thyroxine. This result indicates that extrathyroidal synthesis of thyroxine from injected iodide takes place, the effects observed corresponding to the formation of about 0·12 microgram of *l*-thyroxine from 1·0 milligram of iodine injected as iodide daily.

(6) The administration of thiourea abolishes extrathyroidal synthesis from injected iodide.

(7) Thiourea when administered to rats with intact thyroids does not produce the same degree of thyroxine deficiency as is produced by surgical thyroidectomy.

The retention of the pituitary acidophil cell granules indicates that thyroxine synthesis in the thiourea-treated thyroid gland of the rat is not depressed below one-fifth of the normal rate.

(8) The simultaneous administration of iodide and thiourea to intact animals results in the appearance of the full thyroidectomy changes in the pituitary. This indicates that high iodide concentration acts synergistically with thiourea in depressing thyroxine synthesis in the thyroid gland to insignificant levels.

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THE INFLUENCE OF METHYLATION ON CARCINOGENIC ACTIVITY. I. N-METHYL-3 : 4 : 5 : 6-DIBENZCARBAZOLE.

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BOYLAND and Brues (1937) recorded that the three isomeric, symmetrical dibenzcarbazoles are carcinogenic for the skin of mice, but that, whereas 1 : 2 : 7 : 8-dibenzcarbazole is a weak carcinogen, 3 : 4 : 5 : 6-dibenzcarbazole is much more powerful, 1 : 2 : 5 : 6 having an intermediate degree of activity. They also observed that the painting of 3 : 4 : 5 : 6-dibenzcarbazole on the skin caused bile-duct hypertrophy within a matter of weeks, and frequently also hepatoma in mice surviving 200 days or longer; no metastases were observed from these liver lesions. Strong, Smith and Gardner (1938) confirmed the marked carcinogenic activity of 3 : 4 : 5 : 6-dibenzcarbazole (hereinafter referred to as DBC) when painted on the skin of Cba male mice; according to their section headed "Experimental," they used a stronger solution than did Boyland and Brues, and this may account for the neoplasia of the connective tissue beneath the site of painting which occurred in some of their mice. The great carcinogenic activity of DBC for the subcutaneous connective tissues was demonstrated also by Strong,

Smith and Gardner, who injected the compound in sesame oil, subcutaneously in the right flank, using both Cba mice (liable to spontaneous hepatoma) and A mice (not liable to spontaneous hepatoma). All of 40 Cba male mice developed sarcoma at the site of injection in from 66 to 186 days, and all of 17 A male mice in from 76 to 156 days; in either strain and in mice about 5 months old when injected, the average latent period was a little under 100 days. The liver response in Cba mice painted with DBC was rather less severe than Boyland and Brues (1937) had observed in stock and Simpson albino mice; bile-duct hyperplasia was present, and a low-grade inflammation of the liver was seen, but there were no hepatomas, even of the type which may arise spontaneously in this strain. Injected DBC led to the opposite result, namely, benign hepatomas of the spontaneous type, but occurring earlier than usual, in 8 out of 40 Cba mice, while bile-duct hyperplasia was not seen in any of these livers. In strain A mice, the incidence of liver lesions due to injected DBC followed the pattern for DBC painted on Cba mice, that is, bile-duct hyperplasia was frequent and hepatoma entirely absent; low-grade inflammation of the liver was found in this strain also.

Further experiments with DBC, administered by subcutaneous injection, were carried out by Andervont and Edwards (1941); besides sesame oil, these authors used filtered lard or olive oil as solvent. Mice of strains A, C and C_3H, and, moreover, of both sexes, were used. Whereas Strong, Smith and Gardner (1938) made six weekly injections of 0·2 ml. of 0·1 per cent solution, i.e. a total injection of 1·2 mg. DBC, Andervont and Edwards gave only one such injection, i.e. a total injection of 0·2 mg. DBC. While this in no case led to a 100 per cent incidence of sarcoma at the site of injection, it did enable a comparison to be made between the influence of different solvents and between the susceptibilities of the two sexes, both for tumours induced at the site of injection and in remote sites, i.e. liver and lungs. Andervont and Edwards concluded that sesame and olive oils favoured the induction of sarcoma at the site of injection more than did lard; sesame oil favoured the induction of hepatic changes compared with lard or olive oil. Whichever solvent was used, and in all three strains, there was a much greater susceptibility to hepatic changes in female than in male mice; inspection of the relevant tables shows that hepatic changes appeared much earlier in females than in males. Subcutaneous tumours were slightly more frequent in male mice than in females in strains A and C_3H, while male C mice were much more susceptible than females. The general incidence of subcutaneous tumours was not significantly different for the three strains, but mice of strains A and C were definitely more susceptible to hepatic changes than those of strain C_3H; the latter is especially remarkable, as C_3H mice are known to be more liable to spontaneous liver tumours than are mice of the other strains. No lung tumours were seen in C_3H mice and very few in mice of strain C; hence pulmonary tumours were virtually confined to the strain, namely, A, in which they occur spontaneously. Andervont and Edwards reported only bile-duct proliferation and not hepatoma; this agrees with the findings of Strong, Smith and Gardner (1938) for strain A mice injected subcutaneously with DBC, and contrasts with the findings of these latter workers for CBA mice similarly injected.

From the foregoing account it will be seen that 3 : 4 : 5 : 6-dibenzcarbazole is capable of inducing squamous carcinoma, sarcoma and proliferative hepatic changes in several strains of mice, even in very small doses. This activity is in

sharp contrast with the complete inactivity of the related hydrocarbon, 3 : 4 : 5 : 6-dibenzfluorene (Badger *et al.*, 1940), and with the low activity of the related heterocyclic compound, 3 : 4 : 5 : 6-dibenzacridine (Barry *et al.*, 1935).

Although 4-aminoazobenzene has been shown to be a carcinogen for the liver of rats (Kirby, 1944), it has been known since 1937, when Kinosita obtained many malignant liver tumours with " butter yellow " (N : N-dimethyl-4-amino-azobenzene), that methylation to the dimethyl derivative produced a compound far more carcinogenic for rat-liver than is the parent base. This has also been established for mice of mixed origin (Kirby, 1945*b*). It seemed possible, therefore, that methylation of the imine, DBC, already a very active carcinogen, might further enhance its power and lead to a carcinogen of quite exceptional activity.

EXPERIMENTAL.

1. *Preparation of N : methyl-3 : 4 : 5 : 6-dibenzcarbazole.*

Stevens and Tucker (1923) evolved a method of alkylation for carbazole and certain derivatives; this has proved suitable for alkylation of 3 : 4 : 5 : 6-dibenzcarbazole. Recrystallized from absolute ethanol, N-methyl-3 : 4 : 5 :6-dibenzcarbazole (MDBC) formed magnificent white needles, m.p. 148° C. (sharp).

2. *Painting Experiments.*

The new derivative was submitted to test by painting the skin of stock mice of mixed origin in the interscapular region; a parallel test was made in similar mice with the parent compound, DBC. As Stowell and Cramer (1942) had shown that an acetone solution of methylcholanthrene applied to the skin of mice induced epithelioma more rapidly than did a benzene solution of the same strength, acetone was chosen as the solvent for both DBC and MDBC. Both Boyland and Brues (1937) and Strong, Smith and Gardner (1938) used benzene as solvent for DBC, but whereas Boyland and Brues found a 0·3 per cent solution too toxic for their stock and Simpson albino mice, and had to use a 0·1 per cent solution, Strong, Smith and Gardner were able to apply a 0·5 per cent solution (of the same sample of DBC) for several months to five male mice of the Cba strain. As our experiments were being made with stock mice we began by using a 0·1 per cent solution in acetone of either DBC or MDBC. As, however, no mice exhibited toxic symptoms with either compound, nor had any papillomas appeared in the initial groups by 17 weeks, the strength of the solutions was then increased in both cases to 0·2 per cent, and, after the same interval, in groups begun one to 7 weeks later. Also, 18 weeks after the commencement of the experiment, two groups (each of 6 mice) were set up, using 0·2 per cent solutions of DBC and MDBC respectively from the start. As the initial group of 6 male mice receiving MDBC were accidentally lost after 18 weeks' treatment, painting was discontinued at the same time of the corresponding 6 male mice receiving DBC, which were, however, allowed to live on.

(a) *Skin lesions.*

The survival periods of mice painted with DBC and MDBC and the occurrence of tumours are shown in Fig. 1. Skin lesions are summarized in Table I. The earliest papillomas in the DBC group appeared at 105 days, and one of these was considered malignant on clinical grounds by 150 days. Using a 0·1 per cent solution of DBC in benzene, Boyland and Brues report that the first tumour

appeared at about 170 days, being definitely malignant at 270 days; among the mice painted with 0·3 per cent DBC in benzene up to 125 days and subsequently with a 0·1 per cent solution, these authors reported that a papilloma appeared at about 90 days and an epithelioma at about 180 days. Strong, Smith and Gardner illustrate an epithelioma in a mouse painted with a benzene solution of DBC for 214 days, but give no period for the induction of papilloma. There is some evidence, therefore, that our mice developed skin tumours earlier than those of other workers. Where stock mice were used, as by us and by Boyland and Brues,

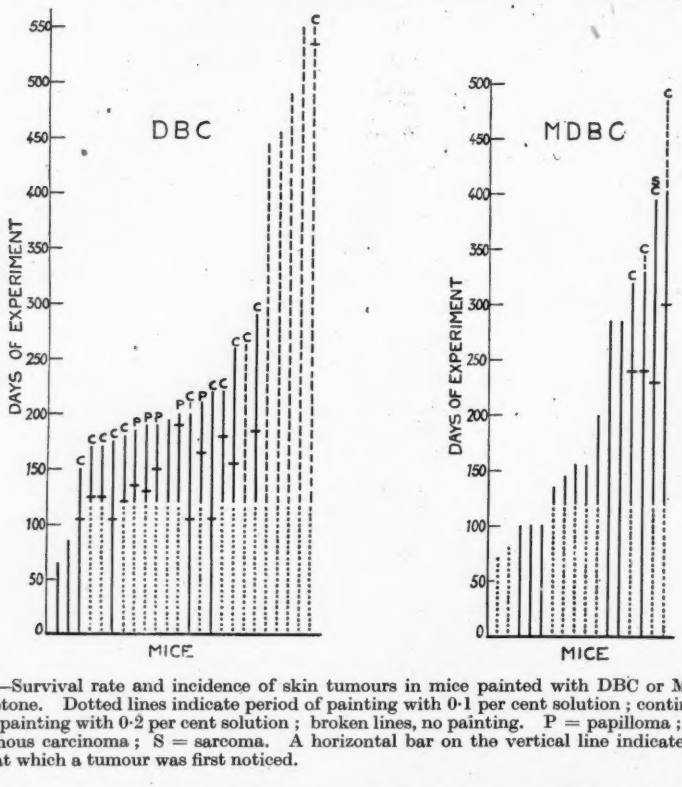

FIG. 1.—Survival rate and incidence of skin tumours in mice painted with DBC or MDBC in acetone. Dotted lines indicate period of painting with 0·1 per cent solution; continuous lines, painting with 0·2 per cent solution; broken lines, no painting. P = papilloma; C = squamous carcinoma; S = sarcoma. A horizontal bar on the vertical line indicates the time at which a tumour was first noticed.

this leads to the conclusion that acetone favoured more rapid development of tumours, both benign and malignant, than did benzene; this accords with the findings of Stowell and Cramer for painted methylcholanthrene.

Moreover, whereas Boyland and Brues obtained tumours in 4/12 mice painted with a 0·1 per cent solution of DBC and surviving over 100 days, and in 8/14 surviving 100 days or more and painted with a 0·3 per cent solution for 125 days and subsequently a 0·1 per cent solution, in our group painted throughout with 0·1 per cent DBC in acetone, 15/16 surviving 100 days' treatment developed tumours at the site of painting by 283 days. Hence it appears that acetone is

more effective than benzene in promoting skin tumours due to DBC in stock mice, both from the point of view of latent period and that of percentage incidence. In our experiment, out of 22 mice painted more than 100 days, 5 developed benign papilloma, while 12 others became malignant; most of the latter were judged on clinical grounds and were subsequently used for therapeutic experiments. Spindle-cell metaplasia was seen in one squamous carcinoma in a male dying at 182 days. Strong, Smith and Gardner reported a sarcoma beneath the site of painting in one mouse and a carcino-sarcoma in another; Boyland and Brues obtained a " spindle-celled epithelioma " in a mouse painted with 1 : 2 : 5 : 6-dibenzcarbazole.

TABLE I.

Lesions.	Painted.								Injected.					
	DBC.				MDBC.				DBC.			MDBC.		
Sex.	M.	F.	?	Total.	M.	F.	?	Total.	M.	F.	Total.	M.	F.	Total.
Number treated over 100 days	11	6	5	22	5	6	3	14	6	6	12	10	5	15
Normal	4	0	0	4	1	3	3	7	0	0	0	9	4	13
Papilloma (benign)	0	2	3	5	0	0	0	0	—	—	—	—	—	—
Hypkeratosis only	0	0	0	0	3	0	0	3	—	—	—	—	—	—
Hyperplasia only	0	0	0	0	2	0	0	2	—	—	—	—	—	—
Malignant tumour (clinical only)	5	3	2	10	—	—	—	—	—	—	—	—	—	—
Squamous-cell carcinoma	2	—	—	2	1	3	0	4	—	—	—	—	—	—
Sarcoma	0	?1	0	?1	0	1	0	1	6	6	12	1	1	2
Invasion	—	—	—	—	1	2	0	3	1	1	2	1	0	1
Parasites	1	0	0	1	0	0	0	0	0	0	0	0	0	0

One interesting feature of our experiments lies in the results obtained by painting 6 male mice with 0·1 per cent solution for 119 days and then with a 0·2 per cent solution for another week; although painting was then stopped, 2 of these mice subsequently developed tumours at the site of painting. Mouse 571 had a definite, broad swelling at the site of painting by 240 days, with no papilloma; by 261 days it was recognizable clinically as a carcinoma. Mouse 576 was killed after 550 days, i.e. 424 days after painting had ceased; it then had a small papilloma at the site of painting, and histological examination showed the presence of a keratinizing tumour considered to be an early squamous carcinoma. If it is assumed that a mouse at $2\frac{1}{2}$ years of age corresponds to an age of 75 years in man, the result in mouse 576 corresponds to a latent period of 35 years *after* an exposure lasting about 10 years, i.e. a total latent period of 45 years.

Contrary to expectation, tumours arose at the site of painting of MDBC in relatively few animals, 4/14 painted for 98 days or more, and at a relatively late period, the first papilloma appearing after 228 days (109 days after a 0·2 per cent solution replaced the initial 0·1 per cent solution) and the first squamous carcinoma after 294 days. Of the four carcinomas, three showed invasion of surrounding tissues but no metastases were found. One female mouse with a keratinizing squamous carcinoma had also a spindle-cell sarcoma, below the site of painting. Two other male mice had hyperkeratosis and hyperplasia at the site of painting and a third had considerable hyperkeratosis without marked hyperplasia.

(b) *Liver lesions.*

All the mice painted with DBC, which were examined *post-mortem*, showed hyperplastic changes in the liver. Of 9 male mice 5 showed bile-duct prolifera-

tion, and in 1 of these the process was neoplastic; 6 of these 9 mice showed nodular hyperplasia of the liver parenchyma, amounting in 2 cases to hepatoma. Three female mice all developed nodular hyperplasia of the liver and one had hepatoma, and bile-duct proliferation was found in 2 cases. Both of 2 mice of which the sex was not recorded had bile-duct proliferation, and 1 had nodular hyperplasia of the liver cells. Thus, of a total of 14 mice painted with DBC, 9 had bile-duct hyperplasia with neoplasia in 1 case and 10 had nodular hyperplasia of the parenchyma amounting to hepatoma in 3 cases. Even in mice painted for only 126 days and then allowed to live on, death ensuing at 443 to 550 days, nodular hyperplasia and hepatoma were found, with haemangiohepatoma in 1 case. Proliferation of the endothelial cells of the liver sinuses was seen in 1 male and 1 female mouse; definite cirrhosis was present in another male and in 1 mouse of unknown sex. A periportal myeloid infiltration was seen in only 1 mouse of each sex. Degenerative changes were common; 3 males and 1 female showed fatty degeneration of the liver, while focal necrosis was present in the livers of 3 males, 1 female and 1 mouse of unknown sex. Mice 573 and 575, males, also showed hyaline degeneration of liver cells in some areas; cholangiofibrosis, a condition classified by Opie (1944) in the livers of rats given N : N-dimethyl-4-aminoazobenzene orally, was found in 1 mouse. One female mouse had a liver abscess.

The livers of the mice painted with MDBC presented a picture much more of degeneration than of proliferative changes. Of a total of 15 mice examined *post-mortem*, only 1, a male, showed bile-duct proliferation, and this mouse was also the only one to show nodular hyperplasia. Moreover, only this same mouse showed proliferation of the sinusoidal endothelium; it also showed definite cirrhosis, but this condition was found also in 1 female mouse. On the other hand, 3 female mice exhibited fatty degeneration of the liver, 4 males and 2 females focal necroses, and 1 male, 1 female, and 2/3 mice of unknown sex, hyaline (waxy) degeneration. One male, 1 female and 2 of unknown sex had abscesses in the liver.

3. *Injection Experiments.*

MDBC was also submitted to test, in parallel with DBC, by subcutaneous injection into stock mice; survival periods are recorded in Fig. 2. It was not possible to get supplies of sesame or olive oil for these experiments, and the solvent used was arachis oil. Although Strong, Smith and Gardner report the injection of 0·2 mg. DBC six times at weekly intervals into Cba mice without toxic effect, Andervont and Edwards (1941) state that two injections of 0·2 mg. DBC at one week interval, using the same concentration in the same solvent, "killed practically every mouse," although it is not clear in which of the three strains they used this preliminary test was carried out. We therefore resolved to begin with a single injection of 0·25 ml. of a 0·08 per cent solution of DBC in arachis oil, i.e. with an injection of 0·2 mg. DBC. This was made in the right flank in each mouse, and a similar injection was made in other mice of a solution of MDBC in the same solvent and of the same strength. With the exception of 6 male mice in the MDBC group, all survivors were injected again after 4 weeks' interval; this injection was made in the left flank and consisted of 0·5 ml. of the original solutions, i.e. 0·4 mg. of DBC or MDBC. Eleven mice in the DBC group and 9

in the MDBC group failed to survive 100 days after the first injection; this evidence of toxicity is in agreement with that of Andervont and Edwards.

(a) *Local lesions.*

These are summarized in Table I. Every one of 6 male mice and of 6 females surviving 16 weeks or more after the first injection of DBC developed sarcomas at one or other, or both, sites of injection; more than one sarcoma arose in the same flank in some mice. Andervont and Edwards report sarcomas due to DBC from 10 weeks onwards in mice of strains A and C_3H. In our experiment 5 mice developed tumours at the first site of injection between 113 and 152 days;

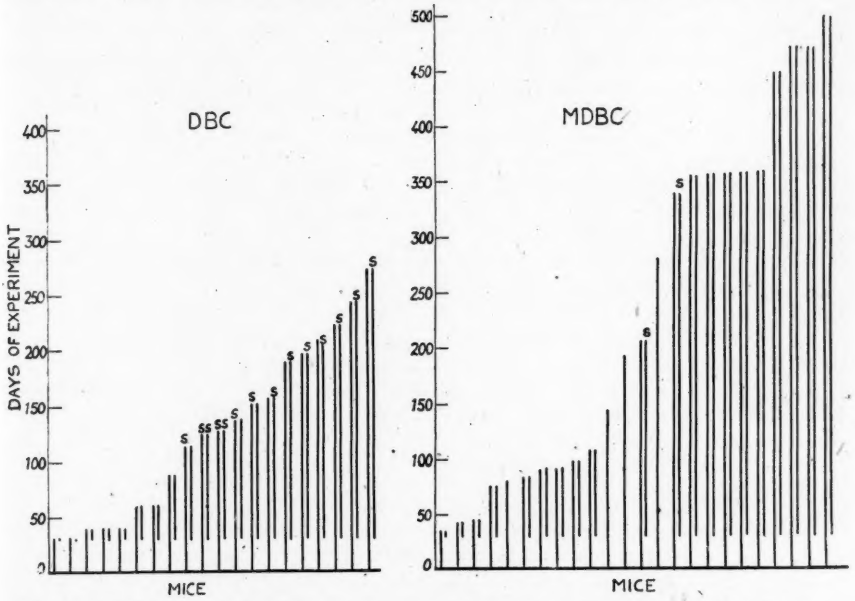

FIG. 2.—Survival rate and incidence of subcutaneous tumours in mice injected with DBC or MDBC in arachis oil. Animals represented by a single vertical line received only one injection in right flank; animals represented by two vertical lines received a second injection later in the left flank. S = sarcoma.

1 dying at 123 and 1 at 126 days had tumours in both flanks, i.e. 89 and 92 days after the second injection. Seven mice surviving 152 days after the first injection failed to develop sarcomas in the right flank, but all of them developed sarcomas in the left flank due to the second injection. Invasion of muscle and adipose tissue was seen in 2 cases, but no metastases were found. Cysts, blue-fluorescent in the U.V. beam, were found in both flanks in mice dying up to 58 days after the first (smaller) injection, and in the left flank (in another mouse) 107 days after the second (larger) injection.

Mice receiving injections of MDBC developed very few local tumours. U.V. fluorescent material certainly persisted at the sites of injection at least as long as in the case of DBC; 1 mouse injected with 0·2 mg. MDBC in the right flank

had a U.V. fluorescent depot when killed 77 days later, and in another, injected in both flanks, there was a blue-fluorescent, milky fluid encysted in contact with an invasive, spindle-cell sarcoma in the left flank, 304 days after the second injection. Nevertheless, beside the sarcoma already referred to, which arose in 1/5 males surviving 100 days after the first injection, only one other local tumour was found; this was a haemorrhagic, spindle-cell sarcoma in the left flank, 171 days after injection, in 1/10 female mice surviving over 100 days after the first injection. No tumours were found in the right flank, i.e. at the site of the first injection, which was only 0·2 mg. Thus it will be seen that MDBC was far less effective than DBC in inducing tumours at the site of injection.

(b) *Liver lesions.*

As in the case of the painted mice, DBC led mainly to proliferative changes, while MDBC caused mainly degenerative changes. 3/9 males and 4/6 females injected with DBC developed bile-duct proliferation of an orderly kind; only 1 male (and no females) showed nodular hyperplasia, but this amounted to trabecular hepatoma and was accompanied by angiomatous sinusoids. No mouse (out of 19) injected with MDBC showed proliferation of bile-ducts or parenchyma, though one showed anisocytosis of liver cells. Both substances elicited proliferation of the sinusoidal endothelium and periportal myeloid infiltration, but these reactions were more frequent after DBC than after MDBC, and only after DBC was cirrhosis found. On the other hand, focal or diffuse necrosis was found in 6/19 after MDBC and in 0/15 after DBC; fatty degeneration in 5/19 after MDBC and in 3/15 after DBC; and hyaline degeneration in 2/19 after MDBC and 3/15 after DBC. No injected mice developed abscesses of the liver. 4/11 males injected with MDBC died after 100 days with normal livers, while 1/9 males and 1/6 females had normal livers at necropsy even after DBC.

Other Lesions Due to Painting or Injection.

(a) *Splenic lesions.*

Changes in the spleen seem to have depended much more upon the route of exhibition than upon the substance administered. Thus when subcutaneous injection was employed, 3/11 spleens were normal after DBC and 5/13 after MDBC; whereas following painting of DBC 1/8 spleens was normal, while all of 10 spleens examined after painting MDBC were abnormal. Degenerative changes were common with either compound. MDBC especially, by either route, but also DBC in 1/8 induced a hyaline (waxy) degeneration, chiefly around the Malpighian bodies, which themselves were usually intact. This hyaline change was reminiscent of amyloid degeneration in human spleen, but the material failed to show metachromatic staining with methyl violet, and took on only a faintly pink stain with Congo red. The appearances resembled closely those described and illustrated by Parsons (1945) following injections of pentose nucleotides in stock mice. Besides hyaline degeneration, both DBC and MDBC induced focal or diffuse degeneration in a few cases.

A common feature of the spleens examined, following administration of either compound by either route, was a lymphoid hyperplasia. Sometimes this accompanied degenerative changes and was presumably compensatory in nature, but usually the hyperplasia occurred in spleens showing no sign of degeneration.

The difference in the incidence between mice treated with DBC and mice treated with MDBC was not significant. On the other hand, where the Malpighian bodies were reduced in size there was usually also degeneration of the pulp. Fibrosis of the spleen was rare and only seen in painted mice.

Two features of the experiment were the number of spleens containing pigmented reticulo-endothelial cells and, more unusual, megakaryocytes. The distribution of the first feature showed no significant difference either for compound or for route of presentation. But megakaryocytes were more frequent in spleens of painted mice than in spleens of injected mice ; there was no significant difference in the incidence of megakaryocytes between the sexes. Without a thorough examination of all the haemapoietic tissues, it would be impossible to assess the significance of the frequent presence of megakaryocytes in these spleens ; the bulk of the pigment was not haemosiderin. Such a result has not followed injection of azo-dyes into mice in our experience, but has been seen following the injection of extracts of the unsaponifiable fraction of liver or bile.

(b) *Kidney lesions.*

About one-third of the kidneys examined in each group were normal. Infection with the parasite *Klossiella muris* was found in 3 female mice painted with MDBC and in 1 male injected with DBC. Oedema was present after painting DBC or MDBC in 1/10 and 1/15 mice respectively ; no injected mice had oedema of the kidney. Hyaline (waxy) degeneration, very reminiscent of human amyloid disease, was not seen in painted mice, but 2/18 mice injected with MDBC showed this lesion ; one of these mice also had the same type of degeneration of the spleen, but the spleen of the other mouse was not examined. Cloudy swelling was present after painting or injection of either substance, but rather more injected mice were affected than painted mice. Similarly, more mice injected with DBC or with MDBC developed an interstitial inflammatory proliferation than did mice painted with either substance. On the other hand, perivascular aggregates of myeloid cells were found, especially around the arciform arterioles, in a larger proportion of painted than in injected mice. Dilatation, degeneration or necrosis of tubules, and the presence of granular casts or debris in convoluted tubules, were occasionally found in either painted or injected mice, and neither substance was more damaging than the other. However, in all these lesions the differences are probably not significant, since the number of mice in each group is too small for statistical analysis.

(c) *Lung lesions.*

Lungs were only examined in 17 animals. In one mouse painted with DBC for 210 days and in one dying 547 days after injection with MDBC (and showing no lesion of liver or subcutaneous tissue) there was a localized lung adenoma. There was no other neoplastic lesion in any lung examined microscopically and no gross lung tumour in any other animal of any group.

DISCUSSION.

The very marked increase in carcinogenic activity, for the liver of rats or mice, which is conferred upon 4-aminoazobenzene by methylating the amino-group (Kinosita, 1937 ; Kirby, 1945*a* ; Miller and Baumann, 1945) is not paralleled by an increase in the carcinogenic activity for skin, subcutaneous tissues or

liver when 3 : 4 : 5 : 6-dibenzcarbazole (DBC) is methylated at the nitrogen atom to form N-methyl-3 : 4 : 5 : 6-dibenzcarbazole (MDBC). On the contrary, MDBC is a considerably weaker carcinogen for mice than is DBC as judged by painting on the skin or by subcutaneous injection. Of 22 mice painted with DBC in acetone over 100 days, 5 developed benign papilloma and 12 others had squamous-cell carcinoma ; of 14 mice similarly painted with MDBC, 4 developed squamous-cell carcinoma. Whereas every one of 12 mice surviving 100 days after subcutaneous injection with DBC in arachis oil developed one or more sarcomas at the site of injection, only 2 of 15 mice similarly injected with MDBC developed a tumour at the site of injection. The tumours caused by MDBC were malignant, as judged by invasion of surrounding tissues.

The capacity of DBC to induce proliferative liver lesions, either of parenchyma or of bile-ducts, almost entirely disappeared on methylation to MDBC. Degenerative changes in the liver were more common following administration of MDBC than of DBC. There was little indication that methylation had made any significant difference to the action of DBC on spleen, kidney or lung.

The marked sex-difference in the incidence of liver changes recorded by Andervont and Edwards (1941) for mice of strains A, C and C_3H injected with DBC was not found in our mixed strain mice similarly injected. As our solvent, arachis oil, differed from any used by the American workers, it is possible that the solvent is responsible for the different results obtained. But it should be pointed out that Andervont and Edwards found essentially an *earlier* incidence in females than in males. A similar claim by Andervont and co-workers (1942) that 4′-amino-2 : 3′-azotoluene induces liver tumours more frequently in female than in male mice has been shown (Kirby, 1945*b*) to be probably rather a matter of earlier response by females.

It is of interest to compare the activities of DBC and MDBC by grading them according to the system devised by Berenblum (1945). Twenty-two mice painted with DBC survived the time at which the first tumour appeared, i.e. 105 days ; 50 per cent of these mice had tumours by 164 days. This gives a " grade of carcinogenicity " of VII, which accords with that given by Berenblum from the data of Boyland and Brues (1937). In the case of mice painted with MDBC, only 6 survived the period required for the first tumour to appear, namely, 228 days ; the third tumour was visible by 241 days. This gives a 50 per cent value of 34 weeks, which corresponds to Grade VI, but as numbers are so small in this group it would be better to designate it Grade 6, as Berenblum does in such cases. This brings MDBC into the same grade as 1 : 2 : 5 : 6-dibenzanthracene and into higher grades than 1 : 2 : 5 : 6-dibenzcarbazole, 1 : 2 : 5 : 6- and 3 : 4 : 5 : 6-dibenzacridines.

Berenblum places DBC as a sarcogen in Grade IX according to the data provided by Strong, Smith and Gardner (1938). The best results of Andervont and Edwards (1941), who used much less DBC per mouse, correspond to Grade VIII. In our experiment with DBC injected subcutaneously, 50 per cent of surviving mice had tumours at the site of injection by 157 days corresponding to Grade VII, but as our data record the time at which the tumour was large enough to leave no room for doubt, the real latent period was certainly less and would correspond to Grade VIII at least. As in the DBC group, so in the MDBC group, 12 mice survived the period, 205 days, required for the first tumour to develop. As, however, only one of the other eleven survivors developed a tumour

at the site of injection, the 50 per cent time-value cannot be found. We are forced, therefore, to adopt the device suggested by Berenblum for weak carcinogens, namely, to multiply the time taken for the first tumour to develop by 2, and use this in place of the 50 per cent time-value. Since our figure of 205 days was for an easily palpable tumour, it would be better to call it 200 days; this gives a value of 400 days for determining the grade of carcinogenicity. The grade for MDBC as a sarcogen is then 4+; this is considerably lower than that for 1 : 2 : 5 : 6-dibenzanthracene, roughly the same as for 1 : 2 : 5 : 6-dibenzacridine, and higher than for 3 : 4 : 5 : 6-dibenzacridine (1 : 2 : 5 : 6-dibenzcarbazole caused no sarcomas at all according to Boyland and Brues).

Thus methylation of the imino-group of 3 : 4 : 5 : 6-dibenzcarbazole still leaves a compound of considerable potency as a cutaneous carcinogen; the sarcogenic activity is reduced much more, but still remains significant.

SUMMARY.

1. N-methyl-3 : 4 : 5 : 6-dibenzcarbazole has been prepared and its carcinogenic and sarcogenic activities compared in mice with those of the parent compound, 3 : 4 : 5 : 6-dibenzcarbazole, by painting in acetone solution on the skin and by subcutaneous injection in arachis oil solution.

2. The results indicate that N-methylation reduces the carcinogenic activity for mice from Grade VII (Berenblum method of grading) to Grade 6 or less. Sarcogenic activity is reduced much more and alters the grading from Grade VIII to Grade 4+.

3. Hyperplastic changes in the liver, which are frequent in mice treated with 3 : 4 : 5 : 6-dibenzcarbazole by either route, are rare in mice treated with the N-methyl derivative.

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FAECAL UROBILINOGEN: CLINICAL EVALUATION OF A SIMPLIFIED METHOD OF ESTIMATION.

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In view of the very gross changes in faecal urobilinogen excretion which occur in pathological conditions, it is evident that in this estimation extreme accuracy is probably unnecessary for clinical purposes, and that a simple method would have many useful applications. The simplified technique presented here was accordingly worked out as a modification of Watson's (1937) method. In the meantime Watson, Schwarz, Sborov and Bertie (1944) have published a somewhat similar modification, but as this employs apparatus or chemicals not readily obtainable in this country at the present time, it was thought desirable to present the accompanying method as an alternative which is perhaps even better suited to routine use. It is applicable to any fresh specimen, and for diagnostic purposes the diagnosis of faecal urobilinogen concentration expressed as mg. for 100 g. of stool is quite adequate. The method may however also be used for 24- and 96-hour collections.

METHOD.

Fresh stool (1·5 g.) is treated with ferrous hydroxide to convert stercobilin pigments to urobilinogen. The filtrate is heated with Ehrlich's reagent and sodium acetate, and the pink colour compared in either a photoelectric colorimeter or in a Duboscq type colorimeter against a neutral grey screen.

Reagents.

(1) 20 per cent $FeSO_4$, 20 g. $FeSO_4$, $7H_2O$ plus 92 ml. of water.
(2) 10 per cent NaOH.
(3) Ehrlich's reagent (Watson), 0·7 g. *p*-dimethylaminobenzaldehyde, 150 ml. concentrated (10 N) HCl, 100 ml. of water.
(4) Sodium acetate solution saturated.
(5) HCl approximately 6 N.

Procedure.

Weigh out 1·5 g. of fresh formed stool into a 6 × 1 in. test-tube. This is readily done by difference from the stool container using a glass rod; 1·5 g. is an amount which will usually adhere to the rod which is finally left in the 6 × 1 in. test-tube. The rough balance used should be sensitive to about 10 mg. 9 ml. of water are then added to the stool and a thorough emulsion produced

with the aid of a glass rod. 10 ml. of $FeSO_4$ are then added and mixed well in, and then 10 ml. of NaOH with further mixing. The mixture is then allowed to stand for one to three hours.

The whole is then filtered through a Whatman No. 1 11 cm. paper, and the rest of the procedure completed without undue delay, which would lead to re-oxidation of urobilinogen.

Two methods of comparison have been used and have given closely similar results.

(*a*) *Photoelectric colorimeter.*

A direct reading one-cell instrument of the Evelyn type as described by King (1942) is convenient, and is now commercially available (Messrs. Gallenkamp). If this is used 2 ml. of filtrate are measured into a dry 250 ml. measuring cylinder. 2 ml. of Ehrlich's reagent are then added and after mixing allowed to stand for 10 minutes. 6 ml. of saturated sodium acetate are then added and the mixture diluted if necessary to a suitable volume with distilled water, according to the amount of pink colour developed. After finally mixing the solution is compared in the colorimeter, using a green filter. A blank solution is prepared at the same time, composed of 2 ml. of filtrate, 2 ml. of 6 N HCl, and 6 ml. of sodium acetate treated as above, and is used for the zero setting.

The instrument is standardized with Terwen's (1925) solution :

1·0 ml. of 0·05 per cent phenolphthalein in alcohol.
5·0 ml. of saturated aqueous Na_2CO_3.
Water to 100 ml.

This gives a colour equivalent to that obtained from 0·387 mg. urobilinogen for 100 ml. according to Watson (1937). The solution should be freshly prepared as it deteriorates appreciably in 1 hour.

Calculation.—Mg. urobilinogen per 100 g. stool $= \dfrac{3{\cdot}87 \times U \times V}{S}$

where U = reading of unknown on logarithmic scale.
S = " standard " "
V = final volume of coloured solution in ml.

The value of S will remain substantially constant provided the same cells and filter are used, so that standardization need only be done occasionally.

(*b*) *Neutral grey screens* (Vaughan and Saifi, 1939).

The chemical procedure here is identical with that given above, except that the blank is omitted. It is therefore rather less precise for very low urobilinogen values. The comparison is made against a screen of suitable density (say 0·5) using a filter with maximum transmission at about 550, the Ilford " spectrum yellow green " being very suitable. This is best done by filling both sides of the colorimeter with a suitable unknown solution to check the lighting, and then replacing one side with water plus the screen. The particular screen and filter combination used is standardized against Terwen's solution prepared as described above.

Calculation.—Mg. urobilinogen per 100 g. stool $= \frac{3{\cdot}87 \times S \times V}{U}$

where V = final volume of coloured solution in ml.
U = colorimetric reading of unknown in mm.
S = ,, ,, Terwen's solution in mm.

Notes on Method.

(1) *Reproducibility of results.*

The degree of reproducibility of the method is within a few per cent, as shown by the following series of duplicate estimation on different 2 oz. specimens :

86, 88 ; 138, 133 ; 292, 272 ; 89, 95 ; 22, 22 ; (mg. per 100 g.).

Larger variations may however occur if complete stools are examined, due to variation in different parts of the stool, and day-to-day variations in one subject may also be considerable, as shown by the following consecutive daily estimation on a convalescent patient :

140, 119, 88, 142, 66, 118, 87, 155, 52 (mg. per 100 g.).

(2) *Age and condition of specimen.*

The stool should be fresh and not liquid in character. Storage for 24 hours in the refrigerator leads to a loss varying from 2 to 46 per cent (average 22 per cent on six specimens).

If stored for 24 hours at room temperature the loss is even greater, averaging 36 per cent (0 to 48 per cent in six specimens).

The presence of blood in the stool does not interfere with the estimation, as the blood pigments are retained in the $Fe(OH)_2$ precipitate. A simple increase in bulk, as in steatorrhoea, may give results slightly below normal. Purgatives do not appear to have this effect, but the method has not been used to liquid stools.

(3) *Estimation of daily excretion.*

In this case it is convenient to collect each stool, or each day's stools, into a large weighed, waxed carton (12 oz. size). The total weight is then obtained by re-weighing the carton plus contents to the nearest gramme, and with the aid of a glass rod several small samples from different parts of the stool are transferred to a smaller carton (2 oz.) and mixed. A second glass rod is now used to remove 1·5 g. from the second carton for estimation as above. Each stool, or each 24-hour sample, is dealt with as far as possible on the day of collection, but it is usually impossible to avoid a 12-hour delay in specimens passed at night time.

(4) *Omission of petroleum from ether extraction.*

Although originally adopted for reasons of convenience this omission appears to be justified by the results obtained, and by the small difference recently reported by Watson *et al.* (1944) for the two methods. These differences were such as would be unlikely to interfere with the interpretation of results, being considerable only in cases with low or normal values. Moreover, the Ehrlich reacting substances not extracted by petroleum ether, and therefore included in the present method, were thought by Watson to be chemically related to

urobilinogen and to have similar clinical significance. The writer feels therefore that any error introduced by omitting petroleum ether extraction is likely to be small in comparison with the known errors due to constipation and collection difficulties referred to above.

However, this omission does lead to the production of small amounts of colour with stools which contain no urobilinogen, when tested by the full Watson technique, and values of 0–2 mg. per cent by the present method are of doubtful significance, and may be reported as " negligible."

RESULTS.

1. *Normal Concentration.*

The faecal urobilinogen concentrations (mg. per 100 g.) in 47 control cases are shown in Table I. These were all adult, afebrile ward patients who were convalescent from diseases which were thought to be unlikely to affect haemoglobin or biliary metabolism, and were made up as follows :

Peptic ulcer, 12 ; chronic bronchitis, 7 ; eczema, 5 ; whitlow, 5 ; diabetes mellitus, 4 ; psychogenic dyspepsia, 3 ; sciatica, 3 ; and miscellaneous, 8. No case with anaemia or recent haemorrhage was included.

TABLE I.—*Normal Faecal Urobilinogen Concentration* (47 *Cases*). (*mg. per* 100 *g.*)

Concentration	0–2	2–14	15–30	31–50	51–100	101–150	151–200	201–220
Number of cases	0	0	3	3	17	15	5	4

Average 101. Standard deviation 56.

It appears from Table I that the normal limits may be taken as from 30 to 220 mg. per 100 g., with occasional cases as low as 15 mg. per cent, the latter probably due to constipation. The average value was 101 mg. per cent.

2. *Normal Daily Excretion.*

Four consecutive 24-hour collections were made in 12 similar convalescent control cases by the method described above in which each 24-hour specimen is separately collected and analysed. The results are shown in Table II.

TABLE II.—*Normal 96-hour Excretions of Faecal Urobilinogen.*

Case No.	Sex.	Age.	Diagnosis.	Highest daily excretion (in mg.).	Lowest daily excretion (mg.).	Average daily excretion (mg.).
1	M.	26	Anxiety state	181	60	121
2	F.	36	Bronchitis	132	0	79
3	F.	35	Mitral stenosis	85	40	73
4	F.	19	Peptic ulcer	173	0	70
5	F.	64	Diabetes mellitus	280	0	70
6	M.	56	Bronchitis	182	0	68
7	M.	22	Impetigo	126	0	64
8	M.	36	Peptic ulcer	70	53	63
9	M.	39	Psychogenic dyspepsia	128	0	58
10	F.	57	Peptic ulcer	74	17	45
11	F.	63	Diabetes mellitus	90	0	30
12	F.	34	Dermatitis	48	0	22

Mean average daily excretion 64. Standard deviation 25.

It will be seen from Table II that the average daily excretion was 64 mg. per day, with variations from 22 to 121 mg. per day on the 4-day collection. Single daily collections naturally showed a wider variation from 0 to 280 mg.

3. *Results in Disease.*

Table III shows the faecal urobilinogen concentration in 76 cases, which are discussed below.

TABLE III.—*Faecal Urobilinogen Concentration in 74 Cases (mg. per 100 g.).*

Diagnosis.	No. of cases.	Normal limits. 0 to 2.	3 to 30.	31 to 100.	101 to 220.	221 to 300.	301 to 400.	401 to 600.	601 to 800.	801 to 1000.	1001 to 1500.
Infective hepatitis	23	1	5	10	4	1	..	1	1	..	..
Bone fractures	12	..	..	..	1	3	1	5	2	..	..
Malaria	11	..	..	..	..	2	3	1	4	1	..
Pernicious anaemia	6	..	..	..	1	3	2	..	..	..	..
Retention jaundice	7	..	..	..	..	..	..	1	1	1	4
Iron def. anaemia	5	..	..	4	1	..	..	..	..	..	..
Obstructive jaundice	10	5	3	2	..	..	..	..	..	..	..

(a) *Obstructive jaundice* (10 *cases*).

Negligible amounts (less than 2 mg. per cent) of urobilinogen were found in 5 cases of complete biliary obstruction, 2 due to gallstones and 3 to malignant disease. In the malignant cases repetition of the test showed no variation from week to week. In addition, 5 cases of partial biliary obstruction gave values varying from 3 to 100 mg. per cent. There is no doubt that sudden relief of obstruction can lead to an excretion rate temporarily above normal (Watson, 1937), although this was not observed in the present series.

(b) *Infective hepatitis* (23 *cases*).

A wide variety of findings is possible in this condition according to the stage and type of hepatitis ranging from low or negligible amounts (6 cases), through the normal range (14 cases), up to a definite increase (3 cases). It was noted that the increased values only occurred during periods of fading jaundice, corresponding to rapid recovery of function and presumably to excretion of retained bilirubin in the bile. The following case will illustrate the point:

	Date. Sept. 14.	Sept. 15.	Sept. 18.	Sept. 20.	Sept. 21.	Sept. 25.
Serum bilirubin mg. per cent	6·4	..	2·7	..	..	1·0
FU. mg. per cent	..	720	280	298	265	161

The opposite finding of continued absence of FU. is illustrated by the following case in a child, aged 4, with a persistent intense jaundice (serum bilirubin 9·8 mg. per cent):

	Date.			
	Nov. 30, 1944.	Dec. 6, 1944.	Dec. 15, 1944.	Jan. 24, 1945.
FU.	Negligible	Negligible	Negligible	6

In this case the appearance of traces of pigment in the stool was the earliest sign of recovery, which was eventually complete.

(c) *Retention jaundice* (7 *cases*).

The number of cases here is small, but the uniformly high results serve to confirm the value of the test in this condition. Three were cases of acquired acholuric jaundice (1430, 1180, 470 mg. per cent), three of chronic haemolytic anaemias of unknown aetiology (1265, 1100 and 860 mg. per cent), and one followed a blood transfusion (625 mg. per cent). In the first three cases repeated estimations gave results of the same high order over a period of some weeks. The effect of splenectomy on the results in one case of acholuric jaundice was as follows:

	Date.				
	July 1.	July 3.		Aug. 3.	Aug. 11.
Serum bilirubin (mg. per cent)	2·8	2·9	Splenectomy	0·4	..
FU. (mg. per cent)	1400	1430		80	293

A further case of acholuric jaundice with an initial serum bilirubin of 2·0 mg. per cent, in which splenectomy was followed by a fatal haemolytic crisis, gave the following results:

	Date.								
	Mar. 21.	Mar. 24.	Mar. 30.		Apr. 5.	Apr. 7.	Apr. 9.	Apr. 19.	Apr. 13.
FU. (mg. per cent)	1180	955	425	Splenectomy	1810	732	587	1100	700

(d) *Malaria.*

In this condition excessive haemolysis is a prominent feature, and the high results are therefore not surprising. All the 11 cases of active (febrile) benign tertian malaria had values above normal, the highest being 904 mg. per cent. None of these cases was jaundiced.

Treatment with mepacrine produced a fall to normal within a few days, as shown by the following:

	Case No.		
	1.	2.	3.
FU. before treatment	904	710	609
FU. after treatment	160	173	115

(e) *Anaemias* (18 *cases*).

In untreated pernicious anaemia 5 out of the 6 cases were above normal, whereas in iron deficiency anaemia all 5 cases were within normal limits and 4 of these were below 100 mg. per cent. These few results serve to confirm the

striking average difference in faecal urobilinogen excretion in these two conditions, as noted by Watson (1937), and suggest that the test is of some value in their differentiation. Effective treatment in pernicious anaemia produces a rapid fall to normal, illustrated by the following cases :

	Date.						
	Oct. 28.		Nov. 7.	Nov. 9.	Nov. 14.	Nov. 17.	Nov. 24.
FU. (mg. per cent)	311	Treatment started	246	204	78	79	74

The 7 cases of haemolytic anaemia were jaundiced and are included under retention jaundice above. They all showed a striking increase in faecal urobilinogen excretion.

In addition to the cases included in Table III, a single case of aplastic anaemia of unknown origin, with a haemoglobin level of 25 per cent (Haldane), was studied. This gave the following results, showing a fall from a high to a normal level during a period in which recovery followed treatment by blood transfusion :

	Date.			
	Aug. 14.	Aug. 22.	Sept. 11.	Oct. 15.
FU. (mg. per cent)	630	692	440	140

The first specimen was obtained before treatment was started, and could not therefore have been due to haemolysis of transfused blood.

(f) *Bone fractures* (12 *cases*).

A number of these were included among those originally chosen as control subjects, with the somewhat unexpected result that they all had values well above the rest of the controls. It soon became evident as previously reported (Maclagan, 1945) that this was a fairly constant finding in any long bone fracture during the first few weeks, with values ranging up to 2–3 times the normal. The 12 cases in Table III were made up as follows :

Femur, 7 ; tibia and fibula, 4 ; and Colles' fracture, 1 ; and were all tested within 2 weeks of injury. Eleven were well above normal, and the twelfth, a case of fractured femur in a woman of 61, gave a value of 210 mg. per cent.

One of these cases, a man with a fractured femur, was selected for further study, and the total daily output of faecal urobilinogen measured by the method described above, with the results shown in Fig. 1 and 2.

After the first 31 days the collections were interrupted for a period of 6 weeks, when a 12-day control period served to establish the normal concentration and excretion rate for this patient. It will be seen from the figures that the output was still above normal at 31 days after the fracture, and the total excess of urobilinogen excreted during this period was 5·67 g., as compared with the excretion during the control period. Some urobilinuria occurred during the first fortnight, but only 0·08 g. were excreted in the urine during the time. The haemoglobin level remained constant at about 80 per cent (Haldane) during the period of investigation.

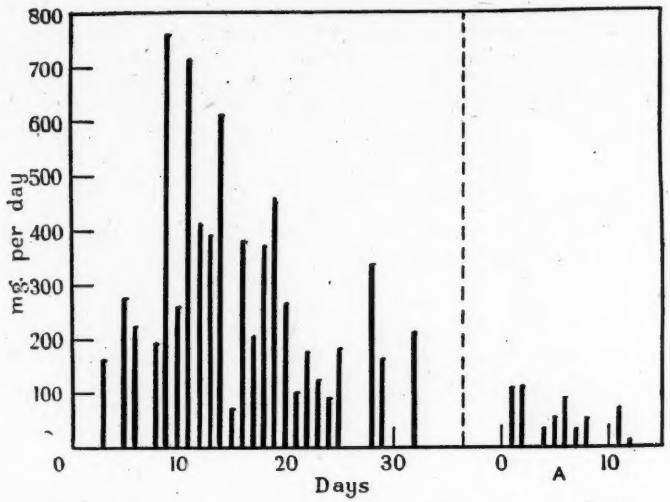

FIG. 1.—Faecal urobilinogen excretion after fracture of femur. A = Control period. Excess excretion = 5·67 g.

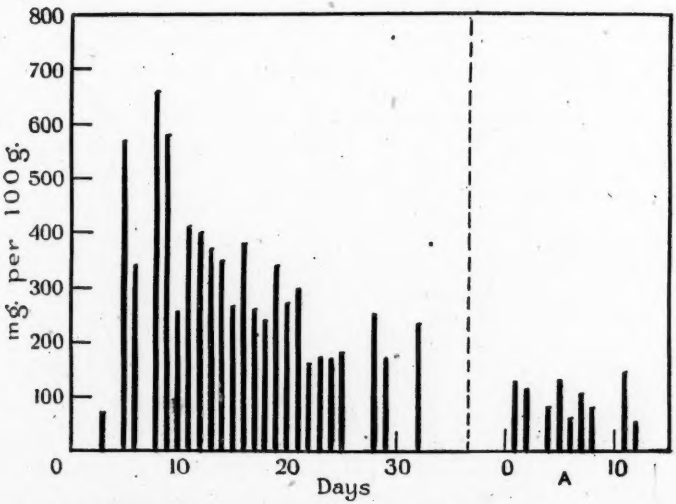

FIG. 2.—Faecal urobilinogen concentration after fracture of femur. A = Control period.

DISCUSSION.

The results presented here indicate that the simplified method described is adequate for the demonstration of those changes in faecal urobilinogen output which are known to accompany disturbances of haemoglobin and biliary metabolism. For many diagnostic purposes the estimation of faecal urobilinogen concentration on any fresh specimen gives all the information required, but the 24-hourly technique is also available for quantitative work. The considerable losses produced by storage emphasize the importance of using only fresh specimens, and also suggest that the bowel habits may at times have an important influence on the results.

The range of values obtained in presumably normal individuals is appreciably lower than that recorded by American workers, as shown in the following table:

Author.	Normal range of urobilinogen concentration (mg. per 100 g.).	Normal range of daily output (96-hour collection).
Watson (1937)	..	40–280
Sparkman (1939)	70–600	..
Watson *et al.* (1944)	72–330	..
Present series	30–220	22–121

No certain reason can be suggested for this discrepancy, which might conceivably be due to difference in the diet, or in bowel habits, or in technique.

The interpretation of the test may conveniently be considered under three headings:

1. *Jaundice.*

The principal value of the test here is in the detection of the retention variety of jaundice, but the temporary increase of FU. which may occur in recovering toxic or obstructive jaundice needs to be considered. This difficulty can usually be resolved by serum bilirubin estimations; a sustained rise of FU. with a stationary or rising bilirubin is good evidence of retention jaundice, while a transitory rise with falling bilirubin is probably due to rapid recovery of hepatic function. In doubtful cases repetition of both tests at intervals of a few days is desirable.

Retention jaundice is used here in the sense of Rich's (1930) classification, and is intended to include accumulation of bilirubin in the blood, due either to overproduction from excessive haemolysis, or to diminished utilization of bile pigments in haemoglobin synthesis. It is not generally realized that the latter mechanism, which operates in aplastic anaemia and to some extent in pernicious anaemia, can produce a disturbance of pigment metabolism apparently identical with that due to excessive haemolysis, except that the jaundice tends to be more intense if active haemolysis is in progress.

With acholic stools the estimation does not always add much to the clinical observations, but the reappearances of traces of pigment in the stool may be the earliest sign of recovery in infective hepatitis, and the presence of blood may change the appearance of such stools. It has also proved of value after operations on the biliary tract in cases where the patency of the bile passages was doubtful.

2. *Anaemias.*

The striking sustained rise of faecal urobilinogen in haemolytic anaemias is of value in diagnosis, and there is also a marked difference between the moderately raised values in pernicious anaemia and the low normal figures in iron deficiency anaemia. A high excretion would be expected in aplastic anaemia, and this was in fact seen in the one case investigated. The uniformly high results in malaria also conformed to expectation. In all these cases the high values fell to normal with successful treatment.

In view of the possible dual mechanism in anaemias discussed above, it is desirable in such patients to consider the results in conjunction with reticulocyte counts. A consistently raised FU. in the presence of reticulocytosis could not be due to non-utilization of bile pigment and must therefore signify excessive haemolysis, as in the chronic haemolytic anaemias. On the other hand, increased FU. without reticulocytosis could be due either to non-utilization, or haemolysis, or both. Thus in malaria there is haemolysis without reticulocytosis in the acute stage (Minot and Castle, 1935; Fairley *et al.*, 1938), and in pernicious anaemia both factors may be concerned (Castle and Minot, 1936; Watson, 1937). In aplastic anaemia non-utilization is presumably the decisive factor.

3. *Bone Fractures.*

The uniformly increased excretion after bone fractures has been briefly reported elsewhere (Maclagan, 1945). The three factors which may be operative here are:

(*a*) absorption of haematomata,
(*b*) non-utilization of bile pigments for haemoglobin synthesis, and
(*c*) excessive haemolysis at some site remote from the fracture.

There is at present insufficient evidence available to permit a final decision as to the relative importance of these three factors, but the possibility of haemolysis remote from the site of the fracture deserves serious consideration in view of the anaemia which is known to follow various types of trauma, and which is thought to be haemolytic in origin (Vaughan, 1945).

SUMMARY.

1. A simplified method for the estimation of faecal urobilinogen is described, applicable to single specimens or to 24-hour collection. Estimations in 120 cases are recorded.

2. A study of the various possible sources of error indicates the importance of testing only fresh specimens.

3. Normal values were from 30 to 220 mg. per 100 g., or from 22 to 121 mg. per day if estimated on 4 consecutive 24-hourly collections.

4. Results were above normal in retention jaundice, malaria, untreated pernicious anaemia, aplastic anaemia, after fractures of long bones, and in certain cases of infective jaundice; they were below normal in many cases of infective and obstructive jaundice.

5. The value of the test as an index of haemolysis is discussed. When used for this purpose it may be desirable to combine the estimation with reticulocyte counts and/or serum bilirubin estimations.

I am much indebted to the medical staff of the Ashford County Hospital for permission to nvestigate their patients, and to Dr. A. G. Signy for access to haematological data. Part of the expenses of the research were defrayed by a grant from Westminster Hospital.

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SOME OBSERVATIONS ON THE BLOOD-CLOTTING MECHANISM. THE ROLE OF FIBRINOGEN AND PLATELETS, WITH REFERENCE TO A CASE OF CONGENITAL AFIBRINOGENAEMIA.

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CLINICAL investigation of individuals who show deviation in behaviour from normal in a single direction has frequently yielded information of physiological value. In a proportion of these cases such studies are the more important because similar conditions cannot be reproduced in experimental animals. We have had the opportunity of studying a patient in the latter category—a girl suffering from a complete absence of plasma fibrinogen. This condition, congenital afibrinogenaemia, is very rare, only seven other cases having been reported ; it has been reviewed by Henderson, Donaldson and Scarborough (1945). Our patient was originally seen by one of us (J. L. P.) in March, 1942, and soon afterwards came under the observation of Witts (1942) and Macfarlane (1945). Subsequently the child has been admitted to St. Thomas's Hospital on several occasions, when the observations reported here were made. These, which appear to shed some light on the behaviour of fibrinogen and platelets, are set out in detail below. Some studies have been made also on the inter-relationship of platelets and fibrin in normal blood coagulation, and on platelets in haemophilia.

MATERIAL.

Case history.—E. E—, female, aged 14. Three days after birth the child bled from her umbilical cord. Since that time her story is one of always bruising easily, punctuated by more serious haemorrhagic episodes. For the treatment of the latter she has been admitted to hospital on no fewer than fifteen separate occasions. The commonest lesion has been bleeding into the tissues of the limbs following some form of trauma. Other phenomena have been haemoptysis at two years, for which no chest lesion could be found, bleeding from the tongue at 4 years during an attack of diphtheria, from a boil at 10 years, and from the tongue after she had bitten it at 11 years. Tooth extractions have also been followed by prolonged oozing of blood. On the majority of occasions these haemorrhagic episodes cleared up sooner or later with absolute rest, with or without local pressure. Sometimes transfusions had to be resorted to, and these stopped the bleeding immediately on each occasion.

Apart from this haemorrhagic tendency there has been nothing remarkable in the child's history. Menses have not yet begun. There are no abnormal physical signs. The child is on the small side of normal and is of average intelligence. She is one of four children of a first-cousin marriage. It is of interest that the mother's brother married the father's sister. None of the patient's relatives have shown any special liability to bleed.

Routine laboratory investigations.—Blood counts have been repeatedly done, and have shown nothing abnormal except anaemia in proportion to haemorrhage. The following examinations were made in June, 1943: Platelets 360,000/c.mm. Coagulation time: Blood still fluid after 5 days. Bleeding time: 3½ minutes. Capillary resistance test negative. Blood sedimentation rate (Westergren): 1 hour, ½ mm.; 2 hours, 1 mm.; 16 hours, 4 mm.; 27 hours, 5 mm. Blood urea: 24 mg. per cent. Serum calcium; 10·6 mg. per cent. Plasma proteins: 8·91 per cent (albumin, 4·82 per cent, globulin 4·09 per cent). Hippuric acid synthesis test: 3 g. sodium benzoate given orally produced the equivalent of 40 per cent of the sodium benzoate in 2 hours in the urine and 78 per cent in 4 hours—a normal response.

Platelet and coagulation time examinations have been made on many occasions before and since that date. The platelets have been reported present in normal numbers on each occasion except once at another hospital, when the total given was 80,000 per c.mm. The coagulation time has always been infinity.

Samples of blood from the father, mother and the two elder children (the youngest being a small baby) have been taken for fibrinogen estimation and all showed figures over 200 mg. per cent.

EXPERIMENTAL RESULTS.

(A) *Establishment of the Fact of Afibrinogenaemia in the Above Patient.*

(i) *Biochemical.*

Micro-Kjeldahl estimation of fibrinogen was impracticable, as no clot was formed on addition of calcium chloride to the diluted plasma. Neither 25 per cent saturation of plasma with ammonium sulphate nor heating the plasma to 55° C. yielded any precipitate. Using a slight modification of the Quick *et al.* (1935) method for estimating "prothrombin" or "accelerated clotting" time, experiments were carried out as indicated in Table I.

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TABLE I.—*Absence of Fibrinogen with Normal Prothrombin in Afibrinogenaemia.*

Normal plasma.	Patient's "plasma."	Fibrinogen solution.	Kinase preparation.	Calcium M 1/40.	Time, secs., to clot.
0·1 ml.	—	—	0·1 ml.	0·1 ml.	29
Defibrinated at 55° 0·1 ml.	—	—	0·1 ml.	0·1 ml.	∝
Defibrinated + 1/10 its vol. fibrinogen solution 0·1 ml.	—	+	0·1 ml.	0·1 ml.	76
—	0·1 ml.	—	0·1 ml.	0·1 ml.	∝
—	+ 1/10 its vol. fibrinogen solution 0·1 ml.	+	0·1 ml.	0·1 ml.	29
—	—	1/10 dilution in saline 0·1 ml.	0·1 ml.	0·1 ml.	∝

Reference to this shows that the following facts were elicited: Normal plasma, with addition of rabbit brain thrombokinase and M/40 calcium chloride in aliquot proportions produced a clot in 29 seconds, whereas the patient's plasma under the same conditions never clotted, though an increase in granularity of brain suspension did occur after 3 minutes. This indicated that at any rate there was no thrombokinase deficiency. Normal plasma defibrinated by heating to 55° C. for 3 minutes likewise produced no clot. When a tenth of its volume of a 3 per cent fibrinogen solution, prepared by salting out from normal plasma with 25 per cent saturation ammonium sulphate, was added to the defibrinated plasma, a clot of normal character was produced in 76 seconds. The increase in time here was due to the partial destruction of prothrombin in the preparation of the defibrinated plasma (Seegers, 1940). As will be seen later, the character of the clot formed is important confirmation of this. When the patient's plasma was treated with the same amount of fibrinogen, a clot was formed similar in consistency to that in the last experiment after 29 seconds. This indicated that the prothrombin in the patient's plasma was at least 80 per cent of normal, and that excess of antithrombin or antiprothrombin was not present. In other words, the clotting defect could be attributable wholly to deficiency of fibrinogen.

(ii) *Serological.*

Antigen-antibody precipitin experiments were attempted in order to see whether by this means fibrinogen could be detected in the patient's blood. Approximately 1 per cent solutions in normal saline of fibrinogen and euglobulin were prepared by salting out from stored plasma with 25 per cent and 33 per cent saturation ammonium sulphate respectively, and dissolving the precipitates so formed in physiological saline. To each of the solutions phenol to 0·25 per cent was added as an antiseptic. Antisera were prepared for these two proteins by injecting the respective solutions in increasing doses intravenously into rabbits and subsequently bleeding them. The doses given were 1, 2, 4, 6 and 8 c.c. on the 1st, 5th, 9th, 13th and 17th days respectively. The rabbits were bled 15 days after the last dose.

Preliminary experiments were carried out to determine the optimum dilutions of antibodies to be used in the precipitin reactions, and a final dilution of 1/5

was found to be satisfactory. Two series of agglutination tubes were set up to contain increasing dilutions of different antigens with each of the two antibodies at the above dilution. Representative results are given in Table II.

TABLE II.—*Precipitin Tests for Fibrinogen.*

A. *Antibody : Rabbit Serum Containing Antifibrinogen. Final dilution* 1/5.

Antigen. Final dilution.	1/2.	1/5.	1/10.	1/20.	1/40.	1/80.	1/160.	1/10,000	Control.
Normal plasma	5+	5+ clot	5+ clot	5+	4+	4+	3+	1+	0
Patient's plasma	1+	1+	1+	—	—	—	—	—	0
1% fibrinogen	2+	2+	3+	3+	3+	4+	3+	—	0
1% euglobulin	1+	1+	1+	1+	1+	1+	—	—	0

B. *Antibody : Rabbit Serum containing antieuglobulin. Final dilution* 1/5.

Patient's plasma	2+	2+	2+	2+	3+
1% fibrinogen	1+	1+	1+	1+	1+

Results read after 5 hours : 5, very heavy flocculent precipitation ; 4, heavy flocculent precipitation ; 3, moderate flocculent precipitation ; 2, fine precipitation ; 1, opalescence.

From this it may be seen that both normal plasma and 1 per cent fibrinogen solution gave strongly positive reactions with antifibrinogen. The fibrinogen and euglobulin solutions reacted weakly with the opposite antisera to dilutions

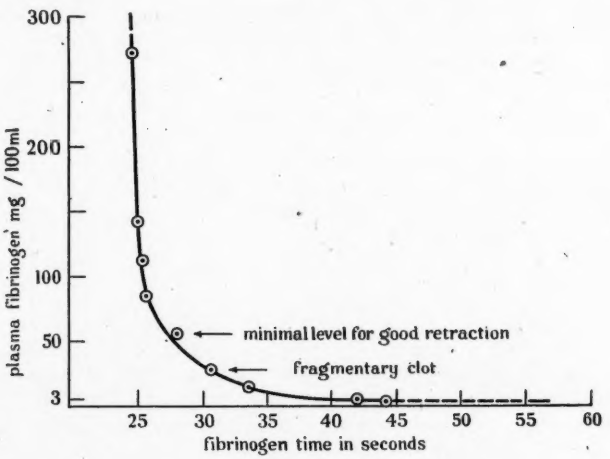


FIG. 1.

of 1/40 and 1/80 respectively. While the patient's serum gave a strong reaction with antieuglobulin, a weak reaction only was obtained with antifibrinogen to a dilution 1/10. As there was a stronger reaction between euglobulin and the same antibody, it was considered reasonable to conclude that no evidence for the presence of fibrinogen could be adduced from these precipitin studies.

The optimal antigen-antibody phenomenon of Dean and Webb (1926) is shown in the reaction between fibrinogen and antifibrinogen and between the patient's plasma and antieuglobulin. The relatively weak response with the fibrinogen solution may have been due to the deterioration of the fibrinogen solution in the refrigerator.

(iii) *Microscopical.*

(*a*) The sediment that formed in the tube mixture in the "accelerated clotting time" estimation of the patient's blood was examined on several occasions, but nothing resembling fibrin was ever observed.

(*b*) Staining reaction: The negative findings in this respect are included in a later section.

(B) *Relation of Fibrinogen Concentration to Clot Formation.*

(i) *In vitro experiments.*

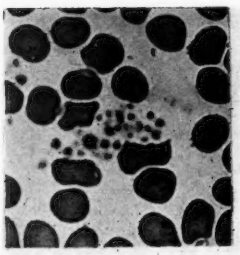
Using the prothrombin time test technique adapted from Quick, Brown and Bancroft (1935), an experiment was conducted to determine the times of coagulation of varying proportions of the patient's and normal plasma. To avoid confusion we have elected to call the accelerated clotting time under these circumstances the "fibrinogen time," this being gauged by the appearance of the earliest visible clot. This definition seems necessary, as it is possible that the moment of initiation of clotting at each dilution occurs at the same time after the addition of the calcium.

The fibrinogen content of the normal plasma was estimated by micro-Kjeldahl technique, so that a relationship could be established between fibrinogen concentration and fibrinogen time (Fig. 1). These figures are confirmatory of those of Witts (1942), who, however, did not estimate the fibrinogen concentrations. The behaviour of clot at each concentration was also observed. Down to a level of 110 mg. per cent fibrinogen, the clot formation was very rapid and retraction satisfactory. At lower concentrations the clot appeared to form increasingly slowly, and in the range 50–25 mg. per cent its retraction was poor. Below the latter figure fibrin formation was only fragmentary. Strands of fibrin could still be seen when the concentration was at the low level of 3 mg. per cent.

(ii) *In vivo experiments.*

On one of the admissions to hospital the patient had bitten her tongue. The bleeding from this proved troublesome. Pressure with local application of adrenaline and dried plasma protein had no effect. Cauterization also failed. The wound was stitched with catgut, which arrested the bleeding. With the subsidence of the inflammatory oedema associated with this operation the bleeding started again. It was decided then to transfuse the patient with dried plasma protein containing 4 per cent of fibrinogen.

The patient was given 300 c.c. of this preparation in approximately $1\frac{1}{2}$ times normal concentration, containing the equivalent of 34 g. total protein (dry weight), during the course of one hour. Apart from a tachycardia for two hours after operation there was no reaction. The bleeding from the tongue had definitely stopped within an hour of the finish of transfusion and the recovery was thereafter



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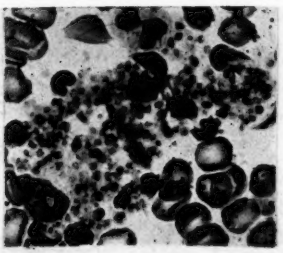

Fig. 3.-

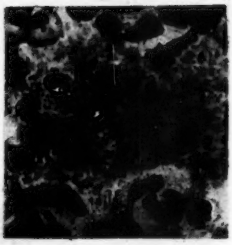

Fig. 4.-

Fig. 5

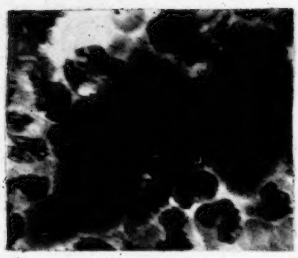

Fig. 6

Fig. 7.-

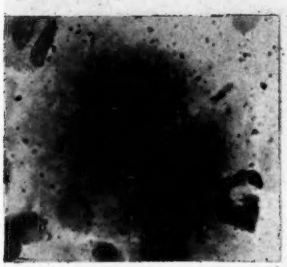

Fig. 8.-

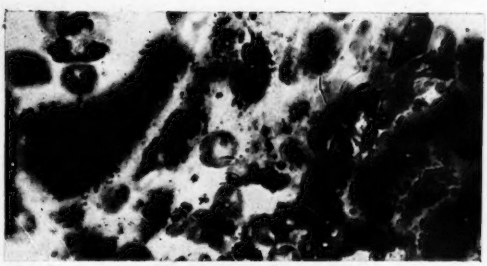

Fig. 9

FIGS. 2–7.—Blood films from the case of afibrinogenaemia, spread at intervals of 1, 3, 4, 5, 10 and 20 minutes after withdrawal. Leishman's stain. × 720.

FIG. 8.—Film of portion of white thrombus removed from side of test-tube in the same case. Leishman's stain. × 720.

FIG. 9.—Blood film from a case of haemophilia spread ten minutes after withdrawal. Leishman's stain. × 720.

uneventful. It is of interest to record that the venepuncture wounds, which previously took a very long time to stop oozing, did not give this trouble in the period immediately after transfusion.

The administration of this plasma gave us the opportunity of studying the fate of the transfused fibrinogen, and of correlating the amount present with the state of the coagulation mechanism at varying intervals of time after transfusion. Samples of blood were withdrawn immediately before transfusion, at the end of transfusion and at intervals of 24, 48, 96 and 192 hours afterwards. On each of these occasions a full blood count, the coagulation time (Lee and White, 1913) and the fibrinogen time were estimated. The corresponding concentration of this protein was calculated from Fig. 1. The character of clot formed, if any, was noted. By the micro-Kjeldahl technique duplicate estimations of the plasma fibrinogen were made. The results obtained are to be found in Table III.

TABLE III.—*Relation of Plasma Fibrinogen, after Transfusion, to Clotting of Blood in Afibrinogenaemia.*

Time from transfusion (hours).	Fibrinogen, mg./100 ml. (micro-Kjeldahl).	Fibrinogen, mg./100 ml. (fibrinogen time).	Clotting time (mins.) (After Lee and White).	Nature of blood-clot.
0	0	0	∞	*Nil*
1	52	33	<8	Well formed, fair retraction.
24	78	>100	5	Well formed, very good retraction, bigger than 1.
48	64	68	5	Ditto
96	30	20	5	Smaller than the last, the retraction good
192	—	3	—	Isolated flakes of fibrin begin to form in 10 min.

From this it will be seen that there is good correlation between the two methods of estimating fibrinogen over the useful range of the " fibrinogen time " technique, and that the latter is the only useful one for the determination of very small amounts of this protein, since under these conditions the micro-Kjeldahl estimation becomes impracticable. It is of interest that, after due allowance had been made for the factor of haemodilution, calculated from haemoglobin values, the fibrinogen did not reach its maximum concentration in the blood until 24 hours after transfusion. An amount of haemostatic significance was still present after 96 hours. The nature of the clot in the withdrawn blood confirms the above findings, and is seen to be of the same order as that obtained in the *in vitro* experiments at similar dilution.

(c) *Observations on the Phenomenon of Platelet Agglutination.*

(i) *Behaviour of platelets in afibrinogenic blood.*

About 10 c.c. of blood were withdrawn from the afibrinogenaemic child into a test-tube and at successive intervals films of this blood were made. These films were fixed and stained with Leishman's stain and examined microscopically under oil (Fig. 2–7). Up to one minute no appreciable change could be seen in the platelets. From that time until the end of the 10th minute progressive alterations could be observed in these bodies. They tended to form clumps of

increasing size, reaching a maximum at about the 10th minute, though the largest number of such clumps were on the 3 and 4-minute films. A cotton-wool-like metamorphosis was observed as early as in the 2-minute film in part of some of the clumps. This appearance later spread to involve whole clumps and became denser and more basophilic in character. The 4-minute film was the first to show this change throughout the substance of all the clumps in the film. With increasing maturity of the metamorphosis the number of chromomeres in the agglutinated masses became smaller, and in the 10-minute film these could not be seen at all. A film made after 20 minutes contained fewer platelet clumps than had been observed at 10 minutes, and they were also smaller, denser, and showed the most intense basophilia.

After three minutes leucocytes began to become enmeshed in the platelet clumps, granulocytes, monocytes and lymphocytes all being involved, and all clumps in subsequent films showed these features.

Four minutes after withdrawal of the blood into the test-tube small white particles, the size of pinheads, appeared on the side of the tube. These increased in number for a few minutes. They were scraped off only with great difficulty from the glass, owing to their adhesive quality. Spread on to a slide and stained in the usual manner with Leishman's stain, these masses were seen on microscopic examination to consist of platelets having the same character as those in the 10-minute film, i.e. they were white thrombi (Fig. 8). Their appearance and increase in number on the side of the tube accounted for the decrease in number on the later blood films. After a week these thrombi were still firmly adherent to the tube, showing no tendency to dissolution.

A similar study was made on oxalated blood from the patient. The behaviour of the platelets here was quite different, the bodies remaining discrete for the whole period of observation. Thus the platelets of this patient were affected by this anticoagulant in the same way as are those of normal individuals.

(ii) *Role of fibrin in platelet agglutination.*

Fibrin and platelets were found not to be birefringent on microscopy, so that dark ground illumination and direct observation of stained films were the only practical courses left open.

(a) *Dark ground microscopy.*—Studies were made on drops of normal blood obtained from the finger and put on clean slides with coverslips at room temperature. Results were generally in confirmation of the findings of Ferguson (1934). By the time the slides were in position on the microscope, platelet agglutination to granular masses had usually occurred ; these were adherent to the surface of the slides and to the under surface of the coverslips. The clumps sometimes showed the linear projections illustrated by Best, Cowan and Maclean (1938), which appeared to have no especial affinity for fibrin. Vesicular excrescences were not commonly observed.

Fibrin needles began to appear here and there in the plasma, usually after about four minutes, and the deposition continued in apparently haphazard distribution for some minutes afterwards. Their rate of formation was slower in thin films than thick. The first appearance was much more frequently in areas which were free from platelets than in the immediate neighbourhood of these bodies. As time progressed there was usually, however, a greater concentration

of needles round the platelet masses than elsewhere, though some clumps were seen, quite a long time after initiation of coagulation, which were either still free from fibrin or showed only a few needles radiating from them.

Study by dark-ground microscopy therefore gave no support to the thesis that fibrin deposition was necessary for platelet agglutination. None could be seen between individual platelets in the clumps, and the fact that there was an interval of about four minutes between agglutination and the appearance of the first fibrin needles—often in areas away from the platelets—makes it unlikely that the substance is laid down earlier on their surfaces.

(b) *Staining reactions.*—Experiments were directed to find dyes which stained fibrin and platelets, and to try if possible to combine stains to produce contrast colouring of the two elements. By this means it was hoped that if fibrin was present on the surfaces of individual platelets it would be possible to visualize it.

Selected dyes were made up in normal saline so that they could be run under the coverslip into clotted blood prepared as for the dark-ground studies. By using this solvent the blood elements kept their natural size, and by using this technique the relations of the various elements in the clot were not disturbed. It was found impossible to get as good fixed preparations under the circumstances, but if the coverslips were removed, the blood dried, fixed with absolute alcohol and then stained, some good results could be achieved.

A number of dyes behaved like methyl violet in staining both elements the same colour. Examples were 1 per cent solutions of Bismark brown (brown), 1 per cent Victoria blue with 20 per cent ethyl alcohol added (blue), 1 per cent picronigrosin (pale green) and methylene blue (blue). In each case the fibrin was stained a paler colour than the agglutinated platelets, i.e. it was rather less basophilic. Two dyes, however, behaved in a slightly different manner.

A 1 per cent solution of soluble indulin in normal saline stained fibrin needles dark grey-black and platelets on the whole were a lighter grey. In studying films to which this dye was added at different intervals of time after withdrawal of blood it was found that recently clumped platelets gave a rather hyaline grey-green colour. The masses appeared quite homogeneous, and showed no evidence of darker striations to suggest initial fibrin deposition. Later, those vesicular, granular and linear metamorphoses which have been described by other authors (Ferguson, 1934; Tocantins, 1938) could be seen, and then the platelet bodies became as dark grey as the fibrin strands.

In the clot fibrin formed a loose woven network over the whole film, only a small proportion of the intersecting points showing platelet clumps. At the circumference of some of the latter the fibrin was rather more plentifully laid down than in the surrounding plasma. Rarely a platelet clump was observed with no fibrin needles in its immediate neighbourhood.

Use of the other dyes gave results similar to those with indulin, though, as stated above, less clearly. Metamorphoses of the platelets were particularly distinct with Victoria blue.

The second dye producing slightly different staining reactions was basic fuchsin. A 1 per cent solution in normal saline containing 20 per cent ethyl alcohol stained platelets a deep carmine, while fibrin strands remained uncoloured or only showed a very faint pink.

In view of this property, shown only by this dye of all those studied, mixtures of it were made in varying proportions with those dyes which stained fibrin,

including indulin. No differential staining of fibrin and platelets could be obtained. The dyes which stained both platelets and fibrin were also mixed in varying proportions, but differential staining was again unsuccessful.

In summary, therefore, staining experiments led to the finding that indulin was the best pigment for the observation of fibrin and platelets. By use of this the conclusion from dark-ground studies was supported.

Films of the afibrinogenic blood spread after 1 minute, and after 20 minutes were stained with methyl violet and with indulin and examined for evidence of fibrin formation. None could be seen either in, around, or at a distance from the platelet clumps.

DISCUSSION

Using several techniques we have been quite unable to detect the presence of any fibrinogen in our patient's blood. Of the methods used, the "fibrinogen time" and precipitin tests would appear most likely to detect the smallest quantities. Considering that as little as 3 mg. per cent produced a readable result in the former test in 40 to 45 seconds, the fact that the patient's plasma gave no result in as long as 3 minutes is strong evidence that only minute amounts could be present in the plasma.

The precipitin tests were clearly not entirely satisfactory owing to the impurity of the preparations used and the antigenic overlap between fibrinogen and euglobulin (Jablonowitz, 1937). The results of Dean and Webb (1926) also indicate that extreme caution is necessary in interpreting the results of such precipitin tests. The data obtained seem to suggest that the maximum concentration of fibrinogen likely to be present in the patient's plasma is of the order of 0·05 mg. per cent, i.e. 1/6000 of the normal amount. These results, taken together with the other chemical tests employed and microscopic examination of blood films, seem to support the view that the "afibrinogenaemia" was virtually complete.

Study of the patient has brought to light observations of interest in connection with the mechanism of haemostasis. Macfarlane (1945) has pointed out that patients suffering from this disease show surprisingly little disability. It would appear to be less incapacitating than haemophilia, in which the coagulation time is often measurable, and it is possible that this difference lies in the behaviour of the platelets. In our patient normal white thrombi were found, and the normal bleeding time and negative capillary fragility tests suggest that there is no interference with capillary contraction in response to injury (Macfarlane, 1941). It is also possible that continuous withdrawal of platelets from the circulation and their replacement by young forms increases the adhesiveness of the platelets present, whereas in haemophilia this adhesiveness is diminished (Wright, 1942, 1946). We have studied the behaviour of shed platelets in the latter disease by the same techniques as those outlined above, and observed that though clumping and fusion of hyalomeres did occur in time, the outlines of the individual platelets in the clumps were distinct at a much later stage than in the afibrinogenaemic (Fig. 9). Also the haemophiliac chromomeres showed comparatively marked stability, up to periods of 10 minutes or more. No white thrombi could be observed in a test-tube after 10 minutes. All these findings indicate slow agglutination and disintegration.

Although the haemophiliac has the mechanism of fibrin formation which is lacking in afibrinogenaemia, clot retraction is delayed on account of the slowness of the agglutination and breakdown of platelets (Tocantins, 1936). Some authors consider that platelets only function in normal haemostasis in initiating fibrin formation and in syneresis (Macfarlane, 1941), but Quick (1942) points out that the " clou hémostatique " in the control of haemorrhage is probably underestimated, and we would therefore support the latter view, especially as far as small wounds are concerned.

The theories concerning the mechanism of platelet agglutination have been summarized by Quick (1942) and Tocantins (1938), and the view postulated by Lenggenhager (1936) that a deposition of fibrin on the platelet is the important factor has gained support (Wright, 1946). The evidence obtained by us is, however, definitely against this view, although it does not absolutely preclude the possibility of the presence of very minute amounts of fibrinogen in our afibrinogenaemic patient. Such small amounts would need to have an extraordinary predilection for adsorption on platelet surfaces, and even this idea is contrary to our microscopic observations. It seems quite possible that some other protein constituent of the plasma participates in platelet agglutination, (Abramson, 1928)—perhaps that fraction found deficient in haemophiliac plasma by Pohle and Taylor (1937).

The experiments on " fibrinogen time " indicated that as little as 50 mg. per cent fibrinogen in the plasma is associated with formation of normal clots showing good retraction, but less than this amount gives friable and fragmentary clots. Hence it seems that fibrinogen must be reduced to less than 15 per cent of its normal value before haemorrhage becomes pathological. This is well correlated with the findings in the afibrinogenaemic after transfusion. Some methods of prothrombin determination involve considerable dilution of the plasma with the object of increasing sensitivity (Shapiro, Sherwin, Redish, and Campbell, 1942). In some instances such dilution may therefore introduce errors in the technique on account of the low fibrinogen content of the diluted specimen.

Concerning the treatment of afibrinogenaemia by replacement therapy, it is a fortunate circumstance that fibrinogen appears to be retained selectively in the plasma. Thus, after 96 hours the plasma fibrinogen was still 30 mg. per 100 ml. (Table III), although the albumin and globulin values had, of course, returned to normal. This finding is contrary to that of Hayward and Jordan (1942) that, in normal patients, the various protein fractions, including fibrinogen, are removed from the plasma at the same rate after transfusion.

SUMMARY.

1. Biochemical, serological and microscopical investigations all suggested complete absence of fibrinogen in the plasma of a case of afibrinogenaemia.

2. Macroscopic and microscopic observations on the platelets in this disease supplied evidence for the normal formation of white thrombi, contrasted with a delay of such formation in haemophilia. This would indicate the " clou hémostatique " is of greater importance in the control of haemorrhage from small wounds than is sometimes thought to be the case.

3. Microscopy of normal blood films also lent no support to the view that fibrin plays a role in the formation of white thrombi.

4. Transfusion of reconstituted dried plasma protein into the afibrinogenaemic patient led to a good retention of fibrinogen, with a corresponding benefit to the haemorrhagic tendency.

Our thanks are due to Dr. Heward Bell for granting us access to the case of afibrinogenaemia; to Dr. J. F. Loutit for his help in supplying the dried plasma of high fibrinogen content; and to Mr. A. E. Clark for help with the photomicrographs.

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SURVEY OF PAPERS.

BIELSCHOWSKY has compared the incidence in piebald and in Wistar rats of tumours produced by the oral administration of 2-acetyl-amino-fluorene. Mammary cancer is more frequent in Wistar rats, while tumours of the ductus acousticus externus and intestinal tumours are more frequent in piebald rats (p. 135).

WILKINS, continuing his search for fungal species that produce antibacterial substances, has examined 100 basidiomycetes and finds that about 10 per cent are strongly positive (p. 140).

SELBIE finds that Shope papilloma virus and sheep dermatitis virus show mutual interference when they are inoculated on the same area of skin of the domestic rabbit, and in certain conditions the resultant papillomas may contain both viruses in a virulent form for long periods after inoculation. Prolonged pre-treatment of the skin with a carcinogenic hydrocarbon has an inhibiting effect on both virus infections, whereas a short pre-treatment with croton oil has a stimulating effect (p. 143).

BECK has studied the effect of feeding mice with carcinogenic hydrocarbons dissolved in aqueous soap solutions. Papillomas and carcinomas arising from the squamous epithelium of the fore-stomach were obtained, but no tumours occurred in the glandular part of the stomach (p. 155).

DICKER, HELLER AND HEWER have investigated the kidney function of rats fed on protein-deficient vegetable diets, and find that they differ from normal rats in that the glomerular filtration rate is correlated with the rate of urine flow and there is a reduction in the total tubular excretory mass. In many of the rats these changes in kidney function are associated with lesions of the renal tubules (p. 158).

PURVES AND GRIESBACH have investigated the relationship between extreme thyroxine deficiency and degranulation of the acidophil cells of the pituitary. Degranulation occurs only in complete thyroid deficiency and can be prevented or restored by injections of thyroxine. Large doses of iodide have a protective action which can be annulled by thiourea, indicating that synthesis of thyroxine may take place outside the thyroid (p. 170).

KIRBY AND PEACOCK find that N-methyl 3 : 4 : 5 : 6-dibenzcarbazole is less active in mice than the parent compound, 3 : 4 : 5 : 6-dibenzcarbazole, in producing tumours and hyperplastic changes in the liver (p. 179).

MACLAGAN has introduced a simplified method for estimating faecal urobilinogen and describes his results in 120 cases. The test is of value in the detection of retention jaundice and in the differentiation of anaemias (p. 190).

PINNIGER AND PRUNTY have investigated a case of afibrinogenaemia. Observations on the blood of this case show that the platelets form white thrombi without the intervention of fibrin (p. 200).